THE MANIPULATION OF FLOWERING TIME TO PRODUCE LEMON FRUIT OUT OF SEASON

By

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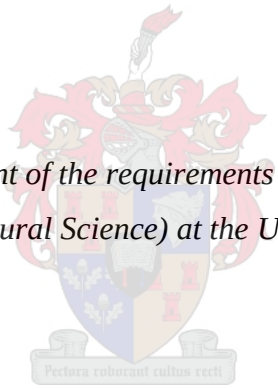

Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Agriculture (Horticultural Science) at the University of Stellenbosch

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Neil Krogscheepers
March 2020

SUMMARY

South African lemon (*Citrus limon*) plantings have increased considerably during the past few years, leading to a possible oversupply of fruit in winter and subsequent declines in current export prices. To address this potential problem, an attempt was made to shift the major lemon fruit harvest to summer, a period during which prices are traditionally higher. This was done by physiologically manipulating the natural flowering habit of a lemon tree, firstly, through an inhibition of spring flowering with the application of foliar gibberellic acid (GA_3) sprays during the floral induction period in autumn; and, secondly, to stimulate a late-summer or autumn flowering response through induced water-deficit stress in mid-summer in a manner similar to the Sicilian *forzatura* technique. An additional objective of the study was to evaluate the effect of these horticultural manipulations on the expression of key citrus flowering genes to shed light on how the reproductive processes are expressed at a molecular level. The lowest concentration of GA_3 foliar sprays, viz. 10 $mg \cdot L^{-1}$, consistently reduced spring flowering compared with untreated trees and to the same extent as the 20 and 40 $mg \cdot L^{-1}$ treatments. Vegetative growth was stimulated in most cases compared with the control. The expression of the *FLOWERING LOCUS T* (*FT*) gene, which is known to integrate floral pathways to initiate the floral cascade, was decreased in buds of trees that received foliar GA_3 treatments. Similarly, mRNA levels of *APETALA 1* (*AP1*) were decreased in GA_3 treated trees. As expected, *AGAMOUS* (*AG*) was not sufficiently expressed to quantify. Adaptation of the *forzatura* technique was however not consistently successful under South African conditions in this study. Varying climatic conditions across seasons and areas resulted in a lack of sufficient water-deficit stress, which translated in non-significant changes in the expression of citrus flowering genes and varying and limited floral induction. In cases where stem-water potential reached levels lower than -2.5 MPa, a significant flowering reaction was observed about three weeks after re-irrigation, similar to Sicilian observations. However, fruit set was very low due to rapid and extensive floral abscission, possibly as a result of low carbohydrate levels and/or wind and insect damage. This study confirmed the efficacy of GA_3 in restricting lemon flowering, while proving that this inhibitive effect is due to downregulation of *FT*. Additionally, the *forzatura* technique was proven to be reproducible in Western Cape production regions, albeit with limited success. Future research focused on increasing fruit set after water-deficit stress conditions may support the practicality and commercial viability of the *forzatura* technique.

OPSOMMING

Nuwe aanplantings van suurlemoene in Suid-Afrika het toegeneem, met die gevolg dat ‘n moontlike ooraanbod van vrugte in die winter bemarkingsvenster kan ontstaan wat mag lei tot afname in prys. Om hierdie potensiële probleem aan te spreek is ‘n poging aangewend om die verwagte suurlemoen produksiepiek in die winter te skuif na die somer, ‘n periode waar pryse tradisioneel hoër is. Hierdie is gedoen deur om die natuurlike blompatroon te manipuleer. Twee afsonderlike eksperimente is uitgevoer om suurlemoenproduksie te manipuleer - eerstens was gepoog om die tradisionele lenteblom te inhibeer met behulp van gibberelliensuur (GA_3) blaarspuite tydens blominduksie in die herfs en, tweedens was die Siciliaanse *forzatura* tegniek aangewend in mid-somer om ‘n blomreaksie in laatsomer of -herfs te stimuleer deur die induksie van gereguleerde vogstremming. Verder was die effek van hierdie hortologiese manipulasies op die uitdrukking van bekende sitrus-blomgene geëvalueer, om meer lig te werp op die geaffekteerde reproduktiewe prosesse op ‘n molekulêre vlak. Die laagste konsentrasie van GA_3 blaartoedienings, naamlik 10 $mg \cdot L^{-1}$, het lenteblom deurlopend inhibeer in vergeleke met die kontrole, met n soortgelyke intensiteit as die 20 en 40 $mg \cdot L^{-1}$ GA_3 behandelings. In die meeste gevalle is vegetatiewe groei in die lente ook gestimuleer vergeleke met die kontrole. Die uitdrukking van die geen *FLOWERING LOCUS T* (*FT*), wat verantwoordelik is vir die integrasie van blom padweë om die blomontwikkelings proses te inisieër, was verlaag in knoppe van bome wat GA_3 toedienings ontvang het. mRNA vlakke van die geen *APETALA1* (*AP1*) was soortgelyk onderdruk as die *FT* geen in behandelde bome. Soos verwag, was die uitdrukking van die geen *AGAMOUS* (*AG*) nie voldoende vir kwantifisering. Die poging om die *forzatura* tegniek toe te pas in plaaslike toestande was nie deurlopend suksesvol nie. Wisselende klimaatstoestande tussen seisoene en areas het die induksie van genoegsame droogtestres belemmer, met die gevolg dat geen beduidende verskille in uitdrukking van sitrus blomgene gevind is nie, asook ‘n varierende, beperkte blomreaksie. In gevalle waar stamwater-potensiaal gedaal het tot benede -2.5 MPa is ‘n beduidende blomreaksie nagenoeg drie weke ná her-besproeiing waargeneem, soortgelyk aan die Siciliaanse waarnemings. Vrugset was egter baie laag aangesien blomme spoedig en teen ‘n hoë intensiteit afgespeen het, waarskynlik as gevolg van lae koolhidraat vlakke en/of wind- en insek skade. Hierdie studie het die effektiwiteit van GA_3 om suurlemoen blomvorming te inhibeer bevestig, terwyl dit bewys het dat hierdie inhiberende effek as gevolg van die onderdrukking van *FT* is. Daar is ook bewys dat die *forzatura* tegniek tot ‘n sekere mate in die Wes-Kaap reproduseerbaar is. Toekomstige

navorsing sal egter moet gefokus wees om vrugset te verhoog ná die aanwending van droogtestres om die kommersiële vatbaarheid te toets.

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NOTE

This thesis is a compilation of chapters, starting with a literature review, followed by two research papers. Each paper is prepared as a scientific paper for submission to the *Journal of the American Society for Horticultural Science*. Repetition or duplicates between papers might therefore be necessary.

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CHAPTER 1

General introduction

Citrus spp. is considered as one of the most important fruit crops (Guo et al., 2018), with lemon [*Citrus limon* (L.) Burm. f.] production central to the citrus industry (M'hiri et al., 2018). Global demand for lemon fruit has increased rapidly in recent years, leading to a substantial increase in the price of fresh export fruit (CGA, 2019). Subsequently, South African lemon plantings have increased steadily, with a subsequent rise of 45% in fruit production from 2016 to 2018, and an expected continued increase in production as many young orchards are still to reach full maturity (CGA, 2019). Therefore, fears of a saturated lemon market and dwindling prices have emerged, with the average returns in Rand per ton for South African lemon fruit already having decreased by 20% since 2016 (CGA, 2019).

Reproductive development of citrus buds is induced during a period of stress, particularly continued low temperature or drought (Lovatt et al., 1992). Therefore, lemon trees that are grown in subtropical climates generally have a large flowering reaction in spring after floral induction during the cold or dry winter months, leading to a harvestable crop in the following winter (Chica and Albrigo, 2013; Hake, 1995). An additional lemon flowering reaction is often obtained in late summer or autumn, of which the intensity depends on cultivar as well as soil- and climatic conditions (Saunt, 2000). The ancient *forzatura* technique as practiced in Sicily utilises this flowering ability of lemon trees by accentuating flower induction during summer by regulated water-deficit (WD) stress, to eventually produce a harvestable summer crop, viz., *Verdelli* lemons, which is marketed in a period when supply of fresh lemon fruit is low and prices therefore higher compared with the winter period (Goodall and Silveira, 1981).

Current literature on lemon flowering was evaluated and indicated a potential for the *forzatura* technique to be adapted in other growing areas with a suitable Mediterranean-type climate, viz., cold and wet winter, and dry and warm summer (Davies and Albrigo, 1994; Goodall and Silveira, 1981). Important aspects related to the success of the *forzatura* technique are the specificity of soil- and climatic conditions, as a sufficient WD stress level is required to stimulate a considerable flowering reaction that would translate into a commercial summer crop (Hake, 1995). Additionally, previous studies highlighted that flower abscission and limited fruit set are major limitations to the successful application of the technique (Barbera et

al., 1985). Furthermore, studies that focused on the effect of WD stress treatments on the expression of known citrus floral genes are restricted to two publications (Chica and Albrigo, 2013; Tang, 2017).

Previous research has confirmed the efficacy of gibberellin (GA) as an inhibitor of citrus floral development, although the physiological mechanism is still unclear (Goldschmidt et al., 1997; Monselise and Halevy, 1964). Attempts have been made to evaluate the effect of GA on the expression of known citrus floral genes to shed light on the physiological mechanism of action by which GA inhibits flowering, but contradicting results have been reported (Goldberg-Moeller et al., 2013; Muñoz-Fambuena et al., 2012; Tang and Lovatt, 2019).

The purpose of this study was to evaluate the potential of manipulating lemon tree flowering to stimulate a possible out-of-season crop under local conditions (Western Cape); firstly by inhibiting spring flowering, and, secondly, by inducing late summer or autumn flowering. Additionally, the expression of citrus flowering genes in reaction to these horticultural manipulation techniques was evaluated to elucidate their role in the citrus floral development pathway. Paper 1 investigated the effect of winter GA_3 foliar applications on flowering and vegetative growth of lemon and lime (*C. latifolia*) trees in three different locations, viz. Citrusdal, De Doorns, and Stellenbosch, over two production seasons. In addition, the effect of GA_3 application on floral gene expression was evaluated. In Paper 2, the potential of adapting the *forzatura* technique to South African conditions was evaluated. Four trials were performed in three different locations, viz. Citrusdal, De Doorns and Stellenbosch, over two production seasons. Furthermore, the expression of known citrus flowering genes succeeding WD stress treatment was evaluated.

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CHAPTER 2

Literature Review: Physiology of Citrus flowering and the manipulation thereof, with focus on *C. limon* (lemon)

2.1. Introduction

Citrus spp. is one of the most important fruit crops (Guo et al., 2018). Lemon [*C. limon* (L.) Burm. f.] production plays a significant role in the global citrus industry (M'hiri et al., 2018) and demand for lemon fruit has risen steadily in recent times, causing a substantial increase in lemon fruit prices (CGA, 2019). As a result of larger profit from lemon production, local lemon plantings have increased considerably in recent years (CGA, 2019). Subsequently, a large proportion of South African lemon orchards are young and not yet in production, leading to fears of a possible fruit oversupply of fruit when these plantings will eventually reach full productivity. These fears need to be addressed, either by looking for new markets or by producing lemons outside the traditional main winter supply period, i.e. in summer.

In order to shift the lemon fruit supply peak to summer, the winter lemon harvest would have to be reduced. This can be achieved by inhibiting the spring flowering reaction through the use of cultural practices. Gibberellin (GA) foliar spray treatments have long been known to inhibit spring flowering in citrus species (Goldschmidt et al., 1997). This is achieved by affecting the development of buds before they are committed to flowering (Lord and Eckard, 1987). The intrinsic mode of action is not yet clear in citrus, neither is known which of the specific flowering genes are affected by this treatment in lemon (Tang and Lovatt, 2019).

Besides chemical manipulation, citrus flowering can also be affected by water stress. The *forzatura* technique has been used for many years in the Sicilian lemon industry (Barbera and Carimi, 1988). In this practice, the tendency of lemon trees to flower repetitively throughout the year is manipulated to produce lemon fruit in summer, when market supply is limited. Under subtropical conditions, lemon trees exhibit a small flowering response in autumn. The aim of the *forzatura* technique is to enhance this flowering reaction in order to set fruit which grow throughout the winter and are harvested in summer. These fruit are called *Verdelli* lemons and are harvested green in the summer after a 12 month growing period (Saunt, 2000).

Citrus trees are induced to flower when exposed to low temperatures (LT) and/or water-deficit (WD) stress conditions (Chica and Albrigo, 2013 a, b). When applying the *forzatura* technique, Sicilian producers impose WD stress to lemon trees by curtailing irrigation during the warm summer months until trees exhibit visual wilting symptoms (Barbera et al., 1985). Following this stress period, trees are re-irrigated after which flowering commences three to four weeks later (Crane, 2004). This technique has been perfected by determining effective stem water potentials to induce flowering optimally and without causing lasting damage to trees (Barbera et al., 1985). Additionally, nitrogen (N) -based fertilisation has been found to promote effective autumn flowering, when applied upon re-irrigation (Hake, 1995).

The pattern of gene expression during floral development is well known for the model plant *Arabidopsis* (*A. Thaliana*) (Bowman et al., 1991). Although it is known that citrus flowering genes are to some extent functionally similar to their counterparts in *Arabidopsis* (Chica and Albrigo, 2013a, b; Endo et al., 2005; Nishikawa et al., 2009; Pillitteri et al., 2004a, b; Tan and Swain, 2007), large gaps still exist in literature as to their expression after specific stimuli. It can be of great use to understand where, when and to what extent certain genes are expressed in the citrus floral development cascade, and what can cause their inhibition.

2.2. The lemon

Lemon is ranked third behind sweet orange [*C. sinensis* (L.) Osbeck] and mandarin (*C. reticulata* Blanco) in terms of production in the global citrus industry (Beltrán et al., 2017). Global lemon production currently amounts to approximately 7.3 million tons, (M'hiri et al., 2018). Lemon fruit are not edible in the same sense as most other fruit types, yet boast a great variety of uses, possibly more than any other fruit (Bartholomew and Sinclair, 1951). Lemon fruit are mostly used in beverages, fresh juice, cooking, flavouring and medicinal purposes (De Villiers and Joubert, 2006). This fruit type boasts a rich variety of nutrients, i.e. flavonoids, citric acid, vitamin C and minerals, all providing numerous health advantages (González-Molina et al., 2009). Additionally, lemon juice is frequently consumed as a natural antioxidant substitute for synthetic compounds (González-Molina et al., 2009).

2.2.1. Origin of lemon species

The origin of the lemon and related citrus species is not very well known (Bartholomew and Sinclair, 1951). Lemon trees have not been found growing in the wild, although similar hybrids

have been found occurring naturally in the Punjab region of Northern India (Spiegel-Roy and Goldschmidt, 1996). Lemon is not considered a true species; there is compelling evidence that suggests that lemon is a trihybrid cross, believed to be derived from citron (*Citrus medica* L.), lime (*Citrus aurantifolia* Swing.), and another gene source, possibly Pummelo [*Citrus grandis* (L.) Osbeck] (De Villiers and Joubert, 2006; Sinclair, 1984; Spiegel-Roy and Goldschmidt, 1996). The general consensus among scientists is that the most likely original habitat appears to have been South-East Asia (Bartholomew and Sinclair, 1951), most probably in mountainous areas where sheltered valleys and southern slopes protect trees from cold and dry winds whilst still receiving warm summer monsoon rains (Spiegel-Roy and Goldschmidt, 1996).

2.2.2. Lemon cultivars

Two lemon varieties dominate the international lemon industry, namely 'Eureka' and 'Lisbon' lemon. 'Eureka' fruit have more prominent ridging, rougher rinds and smaller, less marked nipples than 'Lisbon' fruit (Sinclair, 1984). Additionally, 'Eureka' trees have darker, less pointed leaves than 'Lisbon' trees (Sinclair, 1984). The 'Eureka' tree is only moderately vigorous and is significantly smaller than 'Lisbon' trees, therefore it exhibits slightly lower productivity and also higher sensitivity to frost (Saunt, 2000). However, 'Eureka' trees are less thorny and consequently easier to harvest. Furthermore, they mature a large quantity of fruit in late spring to early summer when prices are high, which makes 'Eureka' the most extensively cultivated lemon cultivar (Bartholomew and Sinclair, 1951; Saunt, 2000). Only in Spain, Italy and some other Mediterranean areas is 'Eureka' not the major cultivated lemon variety (Sinclair, 1984). In South Africa, 75% of lemon plantings consist of the 'Eureka' cultivar, whilst 9% of plantings consist of the 'Lisbon' cultivar (CGA, 2019).

Other economically important cultivars planted in South Africa include 'Fino', 'Limmoneira 8A' and 'Genoa' (de Villiers and Joubert, 2006). 'Berna' is the most important variety in Spain and is also grown on a large scale in Algeria and Morocco, but its fruit can tend to be extremely large (Sinclair, 1984). The 'Femminello' group is the most important Italian variety and is characteristically ever-blooming and -bearing, and thus very responsive to forced treatments such as the *forzatura* technique.

2.2.3. Climatic adaptability

Production of lemon and other citrus predominately occurs between 20 and 40 degrees north and south of the equator, where well defined seasons occur during annual growth cycles (de Villiers and Joubert, 2006). Market prerequisites are relatively less critical for lemon fruit than for other citrus fruits; minimum size and juice percentages are the only important harvest indicators. Therefore, lemon production can occur in borderline, frost-free areas, and out-of-season fruit can easily be marketed, which is not possible for other citrus fruits with more stringent market requirements (Monselise et al., 1981). Lemon has a higher sensitivity to frost than other citrus species, and production is therefore restricted to arid or semi-arid subtropical regions with mild winter temperatures (Monselise et al., 1981). In tropical regions close to the equator with high humidity and warm temperatures all-year-round, citrus trees are inclined to flower sparsely, resulting in uneconomical productivity (Hake, 1995). High humidity also increases the risk of fungal infections (Spiegel-Roy and Goldschmidt, 1996).

2.2.4. Global and local geographical distribution of lemon production

Italy was the first commercial lemon producer with the highest lemon and lime output for many years, until countries like the United States started commercial production in the 1970s (Sinclair, 1984). Currently, the largest lemon and lime producers include Mexico (± 2.5 million tons), India (± 2.4 million tons), China (± 2.4 million tons), Argentina (± 1.6 million tons), Brazil (± 1.25 million tons) and Turkey (± 900 000 tons) (CGA, 2019).

South Africa is ranked ninth on the list of global lemon and lime producers, with a total production of ± 450 000 tons in 2018, yet ranks fifth on the list of global lemon and lime exporters (CGA, 2019). The citrus industry in South Africa has always been and still is export-orientated. Lemon and other citrus exports are a valuable source of foreign exchange income and job creation for South Africa (Malan et al., 2018). South African total citrus exports totalled 299 000 tons in 2018, while the largest international exporters, Mexico and Spain, exported volumes of ± 720 000 tons and ± 610 000 tons, respectively (CGA, 2019).

Commercial citrus production in South Africa occurs in many different small, isolated areas that vary greatly in terms of soil type and climate (Wardowski et al., 1986). Lemons have traditionally been cultivated in the warm/hot citrus production regions of South Africa, but have systematically shifted to cooler areas (Wardowski et al., 1986). The largest lemon production areas in South Africa are the Eastern Cape (42% of production area), Limpopo

(32% of production area) and the Western Cape (13% of production area) (CGA, 2019). In South Africa, fruit from each lemon cultivar mature first in the warmer areas and later in the colder areas, allowing the lemon export period to be stretched over a long period of time. Lemons are harvested in the warm/hot areas (Limpopo) in late February until early April, while the colder areas (Eastern Cape) close the lemon export season in July and August (Wardowski et al., 1986). Lemons are on the market from about week 10 to 40, with the highest volumes shipped between weeks 15 to 30 (CGA, 2019) and therefore are in strong competition with countries from South America's East Coast (de Villiers and Joubert, 2006).

Approximately two-thirds of the South African lemon harvest is exported, while the rest is either processed (130 000 tons in 2018) or sold as fresh produce on the local market (18 000 tons in 2018) (CGA, 2019). The biggest export destination of South African lemons is the Middle East (34%). Europe (29%), South-East Asia (12%) and the United Kingdom (9%) make up the majority of the rest of South Africa's lemon export destinations (CGA, 2019).

2.2.5. Current statistics of lemon plantings and production projections

As of 2019, 14 600 ha are utilised for lemon production in South Africa. Of these plantings, 52% consists of lemon trees younger than five years old (Fig. 2.1). Based on this figure, lemon production is expected to rise sharply in the next few years as younger orchards come into production. This strong upward trend in production has already been witnessed in 2018, as the lemon harvest was 43% larger than that of 2016 (CGA, 2019). South African lemon exports in 2018 equalled 226 thousand tons and with the age of current plantings taken into account, exports are predicted to increase to ± 450 thousand tons million cartons by 2021 (CGA, 2019; Lemmer and Moraba, 2017). This may force producers to seek additional export destinations, else prices will most likely fall.

2.3. Phenology of a lemon tree

Lemon trees are evergreen and have the ability to exhibit year-round, uninterrupted photosynthesis to produce a continuous carbohydrate supply (Nishikawa et al., 2007). Trees have a strong vegetative growth habit and must be pruned in order to control their canopy size (Davies and Albrigo, 1994). The juvenility period of citrus trees is relatively long. In some cases, commercially cultivated citrus trees only flower up to 5 years from planting (Iglesias et al., 2007; Nishikawa et al., 2007).

Lemon, citron (*C. medica* L.) and lime (*C. aurantifolia* Christm.) trees have a tendency to repeat reproductive and vegetative cycles (flushes) throughout the year (Barbera et al., 1985; Hake, 1995). Fruit of different developmental stages are therefore frequently found on the same tree at the same time (de Villiers and Joubert, 2006). Under tropical and some subtropical conditions, flowering of citrus trees could be repetitive, unless the external environmental climate synchronises bloom (Lovatt et al., 1988a, 1992). In subtropical regions, lemon trees may exhibit two main flowering periods; a large bloom in spring and a protracted bloom in late summer (Saunt, 2000), although environmental conditions and cultivar determine the extent of this phenomenon. In subtropical citrus-producing areas, low winter temperatures are necessary to induce a flowering response in the subsequent spring (Chica and Albrigo, 2013a). Under these circumstances citrus trees produce flowers during distinct growth flushes when resting or 'dormant' buds on existing shoots produce new vegetative and/or floral shoots (Hake, 1995). These periods of flush usually occur during spring and autumn (Iglesias et al., 2007).

Where flowering is induced by cold, the spring flowering response is dependent on a continuous period of low temperatures at the start of January [for the Northern Hemisphere (NH)] or June [(for the Southern Hemisphere (SH)], and after a month, initial morphological differentiation of flowers will be completed (Barbera et al., 1985). For the promotion of citrus flowering, low temperature (LT) treatment between 10-18 °C during the day and 5-13 °C at night have proven the most effective (Lovatt et al., 1988a; Tang and Lovatt, 2019; Nishikawa et al., 2007; Southwick and Davenport, 1986; Valiente and Albrigo, 2004).A study on 'Satsuma' mandarin (*C. unshiu* Marc.) showed that when trees were exposed to temperatures above 25 °C, vegetative growth continued indefinitely without a flowering response (Nishikawa et al., 2007).

In tropical climates and in the absence of cold, citrus flowering is stimulated by drought conditions, with flowering commencing at the onset of the first rains of the wet season (Chica and Albrigo, 2013a). According to Srivastava et al. (2000), a monthly precipitation of between 100 and 150 mm following WD stress is adequate to induce flowering. Cassin et al. (1969) examined citrus orchards in tropical climates and studied the flowering response to varying periods of WD stress. The study found that flowering commenced between 20 and 28 days after the relief of WD stress. In a similar manner, under subtropical conditions, drought

conditions during the summer can therefore also induce flowering in autumn (Srivastava et al., 2000).

The severity of flowering response to both stimuli, viz. LT and WD stress, is correlated with the length, as well as the intensity of the exposure of trees to the inductive stimuli (Chica and Albrigo, 2013a). For example, in 'Satsuma' mandarin, the number of flowers that developed in response to LT increased with the duration of exposure (Garcia-Luis et al., 1995). Southwick and Davenport (1986) succeeded in increasing flowering slightly by exposing 'Tahiti' lime (*C. latifolia* Tan.) trees to 2 weeks of LT treatment, but achieved maximum flowering only after 8 weeks of LT treatment. Four weeks of LT treatment were necessary to stimulate flowering in 'Washington navel' sweet orange (*C. sinensis* L. Osbeck), with maximum flowering observed after 8 weeks of LT treatment (Lovatt et al., 1988a). Nishikawa et al. (2007) also found 4 weeks of LT treatment to be sufficient to stimulate flowering of 'Satsuma' mandarin, but 10 weeks of LT treatment were required to achieve maximum flowering.

2.4. Flower development in citrus

In adult citrus trees, reproductive buds are borne on hardened vegetative shoots that developed during the previous season's spring shoot flush. However, they can also develop from shoots from the summer flush (younger) or on older shoots (Krajewski and Rabe, 1995). The fate of lateral meristems depends on the status of the shoot apex; floral apices generally give rise to flowers, whereas vegetative apices generally develop into thorns (Lovatt et al., 1992).

2.4.1. Phases of floral development in citrus

Citrus floral development consists of three synchronized developmental phases – induction, initiation and differentiation (Guardiola, 1997). In California and Israel, 'Washington navel' sweet orange trees are induced to flower from mid-November until the end of December and early January. After completion of flower induction, floral initiation of the apical bud occurs in mid-January, whilst floral differentiation follows floral initiation until anthesis in March to April (Goldberg-Moeller et al. 2013, Lord and Eckard, 1985; Menino et al., 2003).

Vegetative buds are induced to flower during a sufficient period of exposure to LT or WD stress. Floral induction occurs when vegetative growth ceases during the winter 'rest' period, when no apparent growth occurs. Vegetative buds consist of three bracts, six or seven leaves

and a shoot apex (Fig. 2.2). During the floral induction period, vegetative buds develop the capacity to flower after the expression of various flowering genes when a flower-stimulating signal (the so-called *florigen*) is translocated from the leaves to the buds (El-Otmani et al., 2000). Defoliation during the floral induction period inhibits flowering in citrus (Muñoz-Fambuena et al., 2012), which provides evidence that the flowering promotor originates in leaves. In fact, Muñoz-Fambuena et al. (2018) proposed that genetic inhibition of flowering in mature citrus trees is related to suppression of flowering gene expression in the leaves.

Floral initiation can be defined as the floral developmental stage during which the vegetative, but floral induced bud is committed to flower, which according to Lord and Eckard (1987) is complete once the flower sepals have formed. This occurs close to bud break, when induced buds form lateral flower primordia in the axils of the bud leaves and sepals at the apex instead of leaf primordia (Fig. 2.2 B). Citrus inflorescences are cymose, which implies that flower initiation first occurs at the terminal end (Lord and Eckard, 1985).

Differentiation occurs when the vegetative meristem develops into a floral meristem through distinct histological and morphological changes (Fig. 2.2 C) (Davies and Albrigo, 1994). The first sign of reproductive bud differentiation is a flattening of the apical dome (Lord and Eckard, 1985). The terminal flower in an inflorescence usually exhibits the strongest growth rate, with development of subtending flowers lagging behind (Spiegel-Roy and Goldschmidt, 1996).

Citrus inflorescences can either be leafless or leafy (Iglesias et al., 2007). Leafless inflorescences consist of a bouquet of flowers and are usually first to appear in spring, whereas a leafy inflorescence emerges later and is characterised by a smaller number of flowers and the presence of leaves (Iglesias et al., 2007). Lovatt et al. (1984) provided valuable support for this phenomenon by showing that bud break and anthesis of leafless inflorescences occur before those of leafy inflorescences. Inflorescences usually terminate with a flower, whilst containing zero to five axillary flowers and zero to five developing leaves (Lovatt et al., 1988a). Leafy inflorescences generally result in the setting of a higher percentage of flowers into fruit (Davies and Albrigo, 1994; Iglesias et al., 2007), which is likely due to an increased rate of net CO_2 assimilation and provision of photosynthates from leaves that surround the flowering buds (Iglesias et al., 2007). According to Spiegel-Roy and Goldschmidt (1996), when vegetative buds are rapidly committed to flower, leafless inflorescences are generally formed. On the contrary, leafy inflorescences generally develop when commitment of the shoot apex to

flowering is delayed, for example by warm temperatures. Subsequently, this leads to reduced axillary flower development, or in some cases the development of a single terminal flower (Spiegel-Roy and Goldschmidt, 1996).

Once floral induction and differentiation is completed, anthesis will occur if favourable temperature and soil moisture conditions are present (Davies and Albrigo, 1994). Lovatt et al. (1984) concluded that the minimum temperature required for anthesis of 'Washington navel' sweet orange is 9.4 °C; which is significantly less than the minimum threshold temperature for vegetative growth. The terminal flower opens first, followed by the most basal flower, whereas the second flower bud to the apical position opens last (Lord and Eckard, 1987).

2.4.2. Genes relating to citrus flowering

The ABC model of flowering was proposed to explain the activity of floral meristem identity genes of a typical eudicot, based on the model plant *Arabidopsis* (Bowman et al., 1991; Coen and Meyerowitz, 1991; Pelaz et al., 2001). According to this model, *APETALA1 (AP1)* and *APETALA2 (AP2)* are the A function genes responsible for the development of the sepals. *APETALA3 (AP3)* and *PISTILLATA (PI)* are the B function genes, whilst *AGAMOUS (AG)* is the C function gene responsible for development of the floral carpels. The AB gene functions combined specify petal development, whilst BC gene functions specify stamen development.

In *Arabidopsis*, the genes *FLOWERING LOCUS T* (*FT), LEAFY (LFY),* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)* are known as floral pathway integrators which can upregulate genes responsible for establishing and maintaining floral meristem identity (Chica and Albrigo, 2013a; Tang and Lovatt, 2019; Mandel and Yanofsky, 1995; Moon et al., 2005; Parcy, 2005). *LEAFY* functions as both an integrator of floral pathways and as a meristem identity gene (Parcy, 2005). The expression of *FT*, mostly in mature leaf tissue, is necessary for the production of a protein which acts as a mobile signal reported to initiate floral morphogenesis (Abe et al., 2005; Horvath, 2009; Parcy, 2005). This mobile signal is transported to the apical meristem via the phloem (Chica and Albrigo, 2013a; Mathieu et al., 2007), where it activates the expression of floral meristem identity genes, *AP1* and *LFY* (Chica and Albrigo, 2013a; Horvath, 2009). *APETALA1* and *LFY* are responsible for starting the floral developmental cascade, starting with the expression of the A function genes (Blázquez et al., 1997; Bowman et al., 1993).

It has been proven that the homologs of these genes in citrus and other woody species are functionally equivalent to their counterparts in *Arabidopsis* (Endo et al., 2005; Nishikawa et al., 2009; Pillitteri et al., 2004a, b; Tan and Swain, 2007). However, the specific regulatory methods of the expression of these genes could vary, since citrus reacts to different stimuli than those of *Arabidopsis* (Chica and Albrigo, 2013a).

Several studies have found the function of the ABC model genes to be similar in their citrus homologs. For instance, when the citrus homologs of *FT, LFY* and *AP1* were overexpressed in young citrus trees, the juvenile period was significantly reduced and flowering was observed at the seedling stage (Endo et al., 2005; Pena et al., 2001; Pillitteri et al., 2004b). Nishikawa et al. (2007) found an increase in the expression of *FT* in buds and leaves of 'Satsuma' mandarin upon exposure to floral-inducing LT treatment. Additionally, the study found elevated levels of *LFY* expression in buds. Similarly, LT treatment of 'Washington navel' sweet orange increased *FT* expression in leaves and *SOC1*, *LFY* and *AP1* in buds (Chica and Albrigo, 2013a, b; Pillitteri et al., 2004a, b). Nishikawa et al. (2009) further substantiated *FT* as being the ultimate flowering gene in citrus, when increased expression of *FT* was observed during the annual transition to flower development. Furthermore, Pillitteri et al. (2004b) found that the expression of *AP1* and *LFY* in 'Washington navel' sweet orange only increased in the final stages of a floral-inductive LT treatment. This concurs with studies reporting *AP1* and *LFY* as floral meristem identity genes in *Arabidopsis* (Blázquez et al., 1997; Bowman et al., 1993).

Lord and Eckard (1987) provided evidence that buds of 'Washington navel' sweet orange became irreversibly committed to flowering once the sepals were formed. Accordingly, the expression of the citrus homologs of the A organ identity genes (*AP1* and *AP2*) would be the expected developmental markers of floral commitment in citrus buds. This was confirmed by Tang and Lovatt (2019) when they applied flower-inhibiting gibberellin sprays to 'Washington navel' sweet orange trees receiving floral-inducing LT treatment. They found that gibberellin sprays prevented the upregulation of *AP1* and *AP2*, resulting in an increased amount of vegetative buds. The expression of *FT*, *SOC1* and *LFY*, however, was not affected.

2.4.3. Factors influencing citrus flowering

Besides temperature and water relations, carbohydrates, phytohormones and nutrition also have an influence on citrus flowering (Davies and Albrigo, 1994). Generally, higher carbohydrate levels are associated with improved fruit set and yield (Iglesias et al., 2007). For example,

branch or trunk girdling during flower induction is known to increase carbohydrate concentration in plant tissue above the girdle and also subsequent floral intensity and fruit set (Iglesias et al., 2007). On the other hand, low levels of carbohydrates in the roots have been found to reduce shoot and flower production in certain citrus species (Davies and Albrigo, 1994), whereas high N status in leaves and buds are correlated to an increase in flowering in others (Lovatt et al., 1988a, b; Menino et al., 2003).

Spiegel-Roy and Goldschmidt (1996) proposed that flower induction by drought and cold temperatures is possibly caused by a reduction in the concentration of phytohormones such as gibberellins and cytokinins, which are produced in large quantities by young, vigorously growing roots. Further evidence for the importance of phytohormones in flowering is supported by literature and commercial practices, demonstrating that the application of GA_3 to citrus shoots inhibits floral induction and subsequent spring flowering (Davies and Albrigo, 1994; Goldberg-Moeller et al., 2013; Muñoz-Fambuena et al., 2012). The presence of fruit is a well-known inhibitor of floral induction, and a branch that bears fruit will rarely produce flowers, most likely due to inhibition from fruit-derived gibberellins (Spiegel-Roy and Goldschmidt, 1996). Muñoz-Fambuena et al. (2011) found a decrease in the expression of *FT* in leaves as fruit load increased during the flower induction period. It is also well known that a large number of fruit on trees may lead to a reduced bloom in the following year (Garcia-Luis et al., 1995). Gibberellic acid has also been found to inhibit flowering in other perennials and fruiting trees (Spiegel-Roy and Goldschmidt, 1996).

It has been proposed that the termination of root growth is a necessary prerequisite to flowering of citrus (Lovatt et al., 1988a). This is supported by the fact that flower induction of citrus is stimulated by sub-optimum root growth conditions (i.e. low temperature and WD stress) followed by the restoration of favourable growing conditions (Lovatt et al., 1988a). Additionally, cultural practices that restrict root growth, i.e. girdling, graft incompatibilities causing weak rootstocks, small pots that limit root systems and root pruning further support this theory (Lovatt et al., 1992).

2.5. Gibberellin treatments to inhibit citrus flowering

Gibberellins (GAs) collectively consist of hundreds of different compounds. Some of these compounds have been associated with various aspects of plant growth and development,

including seed germination and development, stem elongation, leaf expansion and floral development (Goldberg-Moeller et al., 2013). In general, GAs are associated with the initiation of growth, and are known to stimulate cell division and -enlargement in vegetative organs (Iglesias et al., 2007). The source of endogenous GAs in citrus trees may be actively growing rootlets, seeds, blossoming spurs or fruit tissues (Goldschmidt et al., 1997).

2.5.1. History of the practice

It has long been known that GAs have an inhibitory effect on flowering of various crops, e.g. pome fruit and stone fruit, but Monselise and Halevy (1964) first tested the effect of GA on citrus flowering. They found that fortnightly GA foliar sprays, beginning in November and ending in January (in the NH), inhibited flowering of 'Shamouti' sweet orange trees. Several other studies emphasised the efficacy of gibberellic acid (GA_3) as well as other gibberellins to inhibit flowering of citrus and are listed in Table 2.1.

Low dosages of GA_3 have been found to effectively inhibit flowering, shifting the balance towards vegetative, flowerless shoots, whilst high dosages can prevent flowering of citrus altogether (Spiegel-Roy and Goldschmidt, 1996). Moss (1970) noted that GA treatment increased the ratio of leafy inflorescences to leafless inflorescences, which increased fruit set in the spring. Subsequently, GA_3 can be applied for the control of alternate bearing in commercial citrus production (Guardiola et al., 1977).

2.5.2. Physiological mechanism of action

It is commonly deliberated whether GA acts endogenously to affect flowering (Goldberg-Moeller et al., 2013). It has been proposed that seeds produce GA, which is transported to the buds to inhibit flowering (Bangerth, 2009). There is evidence that fruit load inhibits flowering through the transport of GA from the fruit, as low levels of endogenous GA are correlated with flower initiation, and GA biosynthesis inhibitors stimulate flowering (Muñoz-Fambuena et al., 2012). Growing root tips are also presumed to produce GA in large quantities (Spiegel-Roy and Goldschmidt, 1996). Therefore, a restriction of root growth due to LTs and/or WD stress may lead to decreased GA transport to the canopy, which could help explain the mechanism with which endogenous GA ties into flowering (Lovatt et al., 1988a; Spiegel-Roy and Goldschmidt, 1996; Srivastava et al., 2000). In contrast, continuous root growth, as observed in the tropics, may inhibit flowering due to an uninterrupted supply of GA reaching the canopy (Spiegel-Roy and Goldschmidt, 1996).

It is also possible that GA indirectly inhibits floral development by affecting other physiological processes (Muñoz-Fambuena et al., 2012). For example, Bertelsen et al. (2002) found that, in apple, GA affects the floral process by delaying bud formation, whilst Guardiola et al. (1982) reported that GA reduces bud sprouting in citrus. Additionally, Boss and Thomas (2002) concluded that vegetative growth in grapes is promoted by GA's, which subsequently reduces assimilates available for reproductive growth.

Determining the period in which GA treatment is most effective in inhibiting flowering, is important to understand the underlying mechanism. Monselise and Halevy (1964) reported that GA sprays inhibited the induction process of citrus, as effectiveness in reducing flowering was maximal in the time in which this process takes place (November to January in the NH). Stander (2018) also determined GA sprays to be most effective in reducing flowering of 'Nadorcott' mandarin during the induction period. Later applications of GA_3, when flower differentiation has already been observed under the microscope, have also been found effective in reverting flowering buds to vegetative buds (Guardiola et al., 1982). Although Guardiola et al. (1977) found the most effective flower-inhibiting GA spray to be in the induction period, they hypothesised that the main mode of action of GA is not the inhibition of floral induction, but rather the inhibition of bud development before visible growth occurs in the spring, as they did not find differences in the number of flowers per inflorescence. They concluded that buds of leafless inflorescences are more sensitive to GA and therefore are less likely to develop and sprout in the spring when high concentrations of GA are present.

Lord and Eckard (1987) concluded that GA_3 application inhibits flowering in citrus by allowing the shoot apical meristem to continue vegetative development, if applied before the shoot apical meristem is irreversibly committed to flowering. Furthermore, they stated that once bud determination is complete (once sepals are formed), exogenous application of GA_3 does not have an effect on flowering (Lord and Eckard, 1987). It has also been suggested that all citrus buds are determined to flower from their transition to the adult phase, but when GA is present flowering will be inhibited (Lovatt et al., 1992). Another hypothesis is that flower induction does occur, but subsequent events in the floral pathway only occur if the concentrations of endogenous GAs are below a certain level (Goldschmidt et al., 1997).

Overall, it can be concluded that the presence of GA throughout citrus floral formation, from the induction period to early development before sepal formation, has an inhibitory effect on reproductive development (Goldschmidt et al., 1997; Lord and Eckard, 1987). This inhibition may be due to several developmental factors during the long reproductive process, as previous authors have found GA applications on different stages of the reproductive process to concur with maximum floral inhibition (Goldschmidt et al., 1997). Applications in early winter most likely reduce spring flowering by inhibiting floral induction, while late-winter applications, when bud differentiation has already commenced, are thought to inhibit bud sprouting (Goldberg-Moeller et al., 2013).

2.5.3. Floral genes affected by gibberellin treatments

Guardiola et al. (1982) hypothesised that a certain stimulus originating from the leaves leads to the expression of the floral bud potential, and GA_3 application inhibits this expression. This effect of GA_3 on flowering could be due to interference of *FT* expression, as *FT* expression stimulates flower induction in citrus, and GA_3 application in the period coinciding with flower induction inhibits flowering. Muñoz-Fambuena et al. (2012) found that the expression of *FT* in leaves of 'Salustiana' sweet orange was reduced after a single GA_3 spray and increased after application of a GA biosynthesis inhibitor. They also found that the expression of *LFY* and *AP1* in the leaves was not altered (Muñoz-Fambuena et al., 2012). Goldberg-Moeller et al. (2013) confirmed these observations when they found decreased expression of *FT* in 'Orri' mandarin trees treated with GA_3 foliar sprays. However, they also found a decrease in mRNA levels of *AP1* in GA_3 treated trees.

On the contrary, Tang and Lovatt (2019) concluded that GA inhibits floral development by affecting only *AP1* and *AP2*, and not *FT*. In their study, they found that four to six weekly applications of GA_3 to LT-treated 'Washington navel' sweet orange trees, had no effect on *FT*, *SOC1*, or *LFY* expression. However, the expression of *AP1* and *AP2* was significantly reduced eight weeks after the first GA_3 treatment. In another study by Tang (2017), 'Washington navel' sweet orange trees were subjected to 8 weeks of WD stress, followed by three weeks of re-irrigation. They applied GA_3 in weeks two to eight of the WD treatment, and again found that the expression of *AP1* and *AP2* was reduced compared to unstressed trees, whilst expression of *FT*, *SOC1* and *LFY* was not affected.

2.5.4. Treatment specifics

Monselise and Halevy (1964) found very few flowers differentiated when 'Shamouti' sweet orange branches were under GA influence for 9 weeks, i.e. four 200 $mg \cdot L^{-1}$ GA sprays every two weeks between 3 November and 20 December, as well as the two weeks of lasting effect after the last spray. Guardiola et al. (1982) were successful in inhibiting flowering of sweet orange trees by applying 100 $mg \cdot L^{-1}$ GA at any time between early November and bud sprouting (in the NH). Moss (1970) found the greatest floral inhibition of 'Late Valencia' sweet orange and 'Washington navel' sweet orange trees when GA was applied in late June or early July (in the SH) at concentrations above 25 $mg \cdot L^{-1}$. He proposed this period to be the induction period. Moreover, work done by Guardiola et al. (1977) proved that five 200 $mg \cdot L^{-1}$ GA sprays during the induction period were highly effective in inhibiting flowering of 'Washington navel' sweet orange and 'Navelate' sweet orange trees. Muñoz-Fambuena et al. (2012) found that a single 40 $mg \cdot L^{-1}$ GA_3 spray, applied during the floral induction period to 'Salustiana' sweet orange trees, reduced the number of flowers per 100 nodes by 72% compared with the control trees.

In the study by Guardiola et al. (1982), it was found that 10 $mg \cdot L^{-1}$ GA sprays were effective in inhibiting flowering of 'Satsuma' and 'Clementine' mandarin. Stander (2018) effectively reduced 'Nadorcott' mandarin flowering with two 40 $mg \cdot L^{-1}$ GA_3 sprays at the onset of winter. In an experiment by Khelil et al. (2013), two GA_3 spray treatments of 20 and 40 $mg \cdot L^{-1}$ were applied in November to 'Eureka' lemon trees. A significant decrease in spring flowering was observed for the 40 $mg \cdot L^{-1}$ treatment. Flower number as well as fruit set for the summer and autumn bloom increased as the concentration of GA_3 applied increased, resulting in a heavier *Verdelli* crop load.

In conclusion, GA concentrations above 25 $mg \cdot L^{-1}$ have been found to be effective in inhibiting flowering of citrus. The most effective time of treatment seems to be during the floral induction period and during floral differentiation, before buds are committed to flowering (once sepals are formed). However, timing of the application is difficult, as all the buds on the tree are never at the same stage of development, and subsequently would not react similarly to the treatment (El-Otmani et al., 2000).

2.5.5. The use of anti-gibberellins

Anti-gibberellins such as triazole derivatives have been found to inhibit GA-biosynthesis and subsequently restrict vegetative growth (El-Otmani et al., 2000). These compounds act by blocking the conversion from ent-kaurene to ent-kaurenoic acid in the GA-biosynthesis pathway (Saos et al., 2002). Uniconazole and paclobutrazol (PBZ) are triazoles most popular for inhibiting GA biosynthesis, and have been successfully tested on citrus trees (El-Otmani et al., 2000). However, reports of negative effects on fruit size and yield, as well as environmental conditions having a strong influence on efficacy have restricted commercial use of triazoles (El-Otmani et al., 2000). Effects of triazole derivatives, such as PBZ, include the reduction in ethylene biosynthesis, and the enhancement of cytokinin and abscissic acid contents (Soumya et al., 2017). As these compounds are GA antagonists, they can be applied to control tree size and restrict the vegetative growth reaction of citrus trees (Smeirat and Qrunfleh, 1989).

Three GA antagonists, Cycocel (a chlormequat chloride compound), B-Nine (a daminozide compound) and benzothiazole oxyacetate, have been proven to increase flowering in lemon trees (Monselise and Halevy, 1964; Monselise et al., 1966). Another growth retardant, 2-chloroethyltrimethyl ammonium chloride (CCC) has been tested successfully as a replacement of the summer drought stress for the production of *Verdelli* lemons (Monselise, 1979). However, Moss (1970), could not find any significant effects of CCC application on sweet orange trees.

Greenberg et al. (1993) evaluated the use of PBZ as a foliar spray or a soil application on ‘Shamouti’ sweet orange trees. They found that the number of pure, leafless inflorescences were significantly higher in treated trees than in control trees. In the same study, PBZ applied to ‘Villafranca’ lemon during spring resulted in a nine-fold increase in mid-summer flowers. Medina-Urrutia and Buenrostro-Nava (1995) found that PBZ soil drenches in June (in the tropics) were very effective in increasing flowering in lime trees. They also found a reduction in vegetative growth parameters, i.e. shoot length, shoot girth and shoot number when PBZ was applied. Smeirat and Qrunfleh (1989) also found a reduction in shoot and internode length when a PBZ soil drench was applied to lemons at concentrations of 500, 1000 and 2000 $mg \cdot L^{-1}$. Moreover, PBZ application caused a significant increase in initial and final fruit set.

Initially, results using GA antagonists on citrus have not been consistent, probably because timing is very crucial as inhibitors need to be present before active GA synthesis to achieve

successful antagonism (Monselise, 1979). Additionally, only a narrow range of species is affected by the various GA antagonists, and the level of endogenous GAs possibly have not always been effectively reduced to levels beneath the threshold of floral initiation (Goldschmidt et al., 1997). Only until recently, with the introduction of PBZ, have results regularly reflected consistent floral promotion, whether applied in a soil drench or a foliar spray (Spiegel-Roy and Goldschmidt, 1996). When using GA-synthesis inhibitors as spray treatments, high spray concentrations are necessary, as is the case with growth retardants in general (Monselise, 1979).

2.6. The *forzatura* technique

The *forzatura* technique has been in practice in Sicily, Italy, for at least a century (Barbera and Carimi, 1988). The objective of this practice is to increase late summer/autumn flowering of trees and produce lemons in the following summer (Goodall and Silveira, 1981). This so-called *Verdelli* fruit can be up to ten times the market value of the winter crop (Raveh, 2008). The *forzatura* technique originates from observations on flowering response by Alfonso Spagna in 1867 when lemon trees were re-irrigated after a period of no cultivation (Barbera et al., 1985). Besides lemon growers in Sicily, the technique is also used by lemon growers in Israel, Spain and California, and by lime growers in Florida and Egypt (Spiegel-Roy and Goldschmidt, 1996; Davies and Albrigo, 1994; Goodall and Silveira, 1981).

2.6.1. Application of the *forzatura* technique

The *forzatura* practice consists of the withholding of irrigation during summer, until wilting symptoms appear which do not cease during the night (Barbera and Carimi, 1988; Barbera et al., 1985).

Under normal conditions, i.e. without any forcing, the *Verdelli* crop is set in August (in the NH) and consists of about 10% of annual production (Calabrese and Di Marco, 1981). *Verdelli* fruit remain on the tree for up to 12 months in Sicily and for 9 to 10 months in Israel, and are picked whilst still green (Saunt, 2000). The fruit have a rough, thick rind, are roundish in shape, and usually have a few seeds which are almost completely aborted (Barbera et al., 1985). The *Verdelli* crop is mostly sold as fresh, light green summer lemons (Maranto and Hake, 1985). When the *forzatura* technique is applied in mid-summer, however, heavy flowering can be attained (Burke, 1951) and a larger *Verdelli* crop can be set if the wilting period is followed by

sufficient irrigation and fertilisation. The time at which irrigation is ceased varies depending on climatic conditions and soil water status. In Sicily, irrigation is withheld in early June and resumed in late July, when soil water content reaches values close to wilting point (Barbera et al., 1985; Hake, 1995). Trees become visually wilted with leaf edges that curl without recovering during the night (Barbera et al., 1985). In some cases, some degree of defoliation can occur (Burke, 1951). Flood irrigation has traditionally been used in Sicily to apply the *forzatura* technique, but drip irrigation has also been deemed suitable (Barbera et al., 1985). The trees will usually flower within 3 to 4 weeks after re-irrigation (Crane, 2004).

The optimum levels of soil or plant WD stress patterns for maximum flower induction has not been determined satisfactorily (Hake, 1995), since a large variety of factors can influence these levels. Barbera et al. (1985) reported that overstressing of trees can have various negative effects, e.g., excessive leaf drop, root damage and/or a high abortion of flowers. They found that severe WD stress can lead to flower abscission rates of up to 64% due to restricted floral development, whereas trees subjected to optimum WD stress levels only abscises about 20% of flowers. Overstressing trees through the *forzatura* technique can also have inhibitory effects on the development of fruit from the spring bloom and the previous summer bloom (Barbera and Carimi, 1988).

Previous studies have attempted to determine the intensity and length of the optimum WD stress treatment to cause sufficient bloom without damaging the trees (Table 1). According to a study done by Calabrese and Di Marco (1981), tensiometers should not be used to determine soil water status when the *forzatura* technique is applied, as the soil water levels reached at the end of the dry period are too extreme for accurate measurement. In studies by Barbera et al. (1981) it was reported that xylem pressure potential could be used as a more accurate tool to regulate the *forzatura* practice.

Southwick and Davenport (1986) found that only 2 weeks of moderate WD stress (-2.25 MPa leaf xylem pressure potential) induced flowering in containerised ‘Tahiti’ lime. However, they also found that severe WD stress (-3.5 MPa) for a period of four to five weeks had a significantly larger effect on flowering compared with the control trees which had a leaf xylem pressure potential of -1.48 MPa. Barbera and Carimi (1988) reported that whilst adequate WD stress levels are necessary for sufficient summer bloom, the severity of the WD stress treatment should not exceed a leaf xylem pressure potential of -2.7 MPa in ‘Femminello comune’ lemon

orchards. They found that 7 and 9 weeks of interruptions of irrigation resulted in greater summer and winter lemon yields compared with a 10-week curtailment of irrigation.

Results from experiments by Lovatt and co-workers (1988a, 1988b) in California showed that 16-year-old 'Frost Lisbon' lemon trees subjected to severe WD stress [pre-dawn xylem pressure potential (PDXPP) < -3 MPa] for 20 days followed by moderate water stress (PDXPP < -2 MPa) for 40 days, bloomed significantly stronger than trees subjected to severe WD stress of shorter duration (PDXPP < -3 MPa for 30 days). Their control trees had PDXPP's of > -1 MPa. Hake (1995) applied the *forzatura* technique to 'Frost Lisbon' lemon trees in California for five consecutive years. Results showed that flowering was maximised compared with control trees (PDXPP > -1 MPa), when PDXPP was maintained by WD stress at a level between -2.1 and -3.0 MPa for a period of between 30 to 50 days. He emphasised the importance of systematically reducing the water-status of trees, rather than exposing trees to sudden drought stress. In Florida, Chica and Albrigo (2013a) found that at least 5 weeks of moderate WD stress, resulting in stem water potential (SWP) of -2 MPa, were necessary to induce flowering in 'Washington navel' sweet orange trees. Tang (2017) withheld irrigation from 'Washington navel' sweet orange trees in California for 8 weeks (SWP of -2.7 MPa), before re-irrigating trees for 3 weeks. Trees subjected to the WD treatment produced 51 inflorescences per shoot, whilst the irrigated and untreated control trees (SWP > -1 MPa) produced only 0.8 inflorescences per shoot.

Goodall and Silveira (1981) found that 'Bearss' lime trees that were weak or diseased before WD stress treatment did produce a heavy bloom, but the flowers did not set properly and a harvestable crop was not attained. They concluded that trees that are to be stressed via the *forzatura* technique should be vigorous and healthy.

2.6.2. Nitrogen fertilisation to enhance the *forzatura* technique

Sicilian lemon producers have perfected the *forzatura* technique by applying ammonia-N-based fertilisers to orchards before resuming irrigation (Barbera et al., 1985). Lovatt et al. (1988a) proposed that N fertiliser increased flowering in stressed trees by enhancing the stress-related accumulation of ammonia-N. They proposed that during stress periods ammonia supposedly accumulates, which causes elevated biosynthesis of polyamines such as arginine. These polyamines are important for meristematic activity during flower bud differentiation.

The studies by Lovatt et al. (1988a, 1988b) proved that the strength of the flowering reaction is related to the N status of lemon trees. An increase in ammonia-N concentration in leaves corresponded to stronger flower induction via LT treatment or WD stress, with trees subjected to the highest level of stress treatment having the highest ammonia-N concentration. Additionally, the flowering reaction could be intensified with N fertilisers when induction was not thorough (i.e. moderate water stress or temperature). Therefore, ammonia-N fertilisation is a possible replacement for severe WD stress. In support of this, Hake (1995) found that foliar application of urea sprays can, to some extent, replace the WD stress in field and container-grown lemon trees. An important observation by Lovatt et al. (1988b) was that low-biuret (LB) urea application to citrus trees exposed to low-temperature treatment increased leaf nitrogen status and the number of flowers per tree but did not increase the number of vegetative shoots produced.

Lovatt et al. (1992) found that yearly winter applications of foliar LB urea to 'Washington navel' sweet orange trees during or preceding flower initiation increased fruit yield significantly for three consecutive years. Hake (1995) showed that a single foliar LB urea application to 'Frost Lisbon' lemon trees significantly increased flowering in trees experiencing moderate WD stress (-2.0 MPa). Additionally, he found that in cases where LB urea application did not increase the ammonia-N status of WD stressed trees to levels beyond that of WD stressed trees not receiving LB urea fertilisation, flowering was not significantly higher. He concluded that, if foliar LB urea treatment is to increase *Verdelli* flowering, average ammonia/ammonium levels in leaves must be higher than those in trees not receiving urea treatment.

The timing of N fertiliser was deemed crucial in an experiment by Ali and Lovatt (1994); foliar application of LB urea to 30-year-old 'Washington navel' sweet orange trees during mid to late winter caused a stronger flowering reaction in the spring compared to trees receiving LB urea earlier in the winter.

2.6.3. Floral genes affected by the *forzatura* technique

According to studies evaluating citrus flowering genes, expression of *FT* is enhanced by LT treatment (Nishikawa et al., 2007; Pillitteri et al., 2004b). This is consistent with literature defining *FT* as the flower promoting signal in *Arabidopsis* (Abe et al., 2005). *APETALA1* and *LFY* expression only increased toward the later stages of low-temperature treatment in an

experiment on 'Washington navel' sweet orange (Pillitteri et al., 2004b). This is consistent with literature defining these genes' homologs in *Arabidopsis* as floral meristem identity genes (Chica and Albrigo, 2013a; Mandel and Yanofsky, 1995). Therefore, *FT* could be expected to be the ultimate flowering gene in lemon and the expression of *FT* will subsequently increase upon WD stress application. Although literature evaluating the effect of LT stress on the expression of citrus floral genes is relatively abundant, similar studies evaluating the effect of WD stress are rare.

Chica and Albrigo (2013a) studied changes in the expression of floral genes when 'Washington navel' sweet orange trees were exposed to WD stress. They recorded the changes in the accumulation of four flower-promoting genes; *FT*, *SOC1*, *AP1* and *LFY*. They found that WD stress resulted in the up-regulation of *FT*, causing an increased floral reaction. The expression of *SOC1*, *LFY* and *AP1*, however, was reduced in the WD stress trees. When trees were re-irrigated, the expression of *SOC1*, *LFY*, and *AP1* increased. Therefore, the authors concluded that WD stress induces flowering through the up-regulation of *FT*.

The only other study done to evaluate the effect of WD stress on citrus floral gene expression was performed by Tang (2017). In this study, WD stress was applied to 'Washington navel' sweet orange trees for 8 weeks and re-irrigated for 3 weeks thereafter. In contrast to the study by Chica and Albrigo (2013a), the author did not find a significantly higher rate of *FT* expression in the buds of WD stressed trees compared to control trees. However, the expression of *AP1*, *AP2*, *SEPELLATA* (*SEP*), *PISTILLATA* (*PI*) and *AGAMOUS* (*AG*) genes were significantly higher in WD stressed trees compared with control trees. *SEPELLATA* and *PI* were never expressed at detectable levels in control trees, whilst *AG* was only expressed at very low levels. Therefore, the author concluded that WD stress induces flowering through the up-regulation of *AP1* and *AP2*.

2.6.4. Drawbacks/disadvantages of the *forzatura* technique

The *forzatura* technique has been applied in Italy for many years and subsequently is adapted to specific climatic conditions. Producers aiming to adapt this technique in other regions should take the local micro-climate into consideration to ensure trees are stressed sufficiently without long-term fruit yields decreasing.

Difficulties experienced when applying this technique include the presence of rainfall prior to sufficient drought stress and/or highly retentive soils, leading to inadequate levels of WD stress and subsequent floral induction (Crane, 2004). This has been experienced in a previous study done by Goodall and Silveira (1981), where an effort was made to adapt the *forzatura* technique to Californian conditions. In this study, a test block had to be withdrawn due to a lack of stress experienced by 'Bearss' lime trees in retentive soils. Burke (1951), upon surveying the Italian citrus industry, stated that the best results were achieved with orchards in light, sandy or gravelly soils. He also concluded that terraced plantings were especially effective, where drying was more thorough, as a larger soil area was exposed to the sun.

On the contrary, heavy water stress may weaken trees and reduce their yield potential (Crane, 2004). Burke (1951) found that excessive water stress jeopardised the life and vigour of lemon trees, causing defoliation, dropping of fruit and sparse flowering. In a study done to evaluate the recovery of 'Valencia' sweet orange trees after severe WD stress, Fereres et al. (1979) found that trees with PDXPP less than -2.5 to -3.0 MPa were weakened considerably, exhibiting a significantly smaller flowering reaction and a reduced harvest upon re-irrigation.

An alternative to completely withholding irrigation is controlled daily deficit irrigation applied during the WD stress floral induction period (Hake, 1995). This may allow trees to slowly reach the desired PDXPP and maintain them at that level of stress for the length of time required for maximum induction, especially in sandy soils.

According to Raveh (2008), a major drawback of the *forzatura* technique is that it can only be repeated in alternate years, as it weakens the trees, therefore, partial root-zone drying (PRD) is a possible alternative. However, Hake (1995), found that tree health, quantified by winter yield, did not decrease in five consecutive years of *forzatura* application.

Other disadvantages of the *forzatura* technique include increased use of fertiliser, greater risk of fruit damage from fungi and insects, increased risk of frost damage to small fruit and possible loss of long-term tree vigour (Maranto and Hake, 1985). Also, too severe moisture stress may lead to internal discolouration and reductions in winter fruit quality (Maranto and Hake, 1985). Barbera and Carimi (1988), however, found no negative effects of summer WD stress treatments on winter fruit quality. An additional potential drawback of the technique is the required growth period. Monselise et al. (1981) stressed the fact that *Verdelli* lemons require

much longer growing periods than fruit originating in spring, and that fruit are much greener due to high temperatures experienced during the colour break period.

. Despite the risks of the *forzatura* technique, Burke (1951) believed that if the practice is adhered to with the utmost care and perfection, damage to trees can be minimal. He substantiated this claim by reporting no injury or weaknesses in Sicilian lemon orchards that had been 'forced' with WD stress for over 40 years.

2.7. Conclusion

Based on the age of current lemon plantings, production in South Africa is expected to continue a strong upward trend (CGA, 2019). This can bring forth a possible oversupply in export markets, leading to reduced prices. It may therefore be necessary to seek additional markets or shift the lemon supply peak to a period of lesser competition via horticultural manipulations.

Gibberellins have an inhibitory effect on citrus flowering; they are highly effective in reducing spring flowering when applied as a foliar spray during the floral inductive period (Table 1). Additionally, winter GA applications can increase the amount of vegetative shoot flushes in the spring (Spiegel-Roy and Goldschmidt, 1996). The physiological mechanism in which GA acts is not clear, although popular hypotheses implicate the inhibition of the bud development of inflorescence meristems (Guardiola et al., 1977), or the inhibition of floral induction before development is initiated (Lord and Eckard, 1987; Lovatt et al., 1992). The effect of GA on citrus flowering is further substantiated by studies proving that gibberellin-synthesis inhibitors can increase spring flowering of citrus trees (Greenberg et al., 1993; Medina-Urrutia and Buenrostro-Nava; 1995).

The *forzatura* technique is an established technique used by Sicilian lemon producers for many years. However, field research on the *forzatura* technique is limited and variable, due to inherent cultivar- and soil variation in producing areas (Hake, 1995). An increased ammonia-N concentration in lemon tree buds correspond to a stronger flowering reaction after inductive WD stress or LT treatments (Lovatt et al., 1988a, b). Therefore, nitrogen fertiliser can be applied to amplify the *forzatura* flowering reaction (Barbera et al., 1985).

Even though a substantial lemon flowering reaction may be the result of the *forzatura* technique, a high rate of flower- and fruitlet abscission may offset any advantages gained by the technique (Fereres et al., 1979; Burke, 1951). There are also risks of long-term damage to trees and shortening of the lifespan of orchards. However, if the practice is correctly applied and trees monitored closely, damage should be minimal (Burke, 1951). Commercial application of the technique can be impractical in areas of variable climactic conditions due to the specificity of the timing of the WD stress period (Crane, 2004). Therefore, orchards with highly reactive light, sandy soils in areas with predictable hot, dry summers are most suited for the *forzatura* technique (Burke, 1951; Crane, 2004).

The genes most active in citrus floral regulation, specifically in reaction to WD stress and GA treatment appears to be *FT* and *AP1*, with various authors correlating their expression to the strength of the flowering reaction (Muñoz-Fambuena et al, 2012; Chica and Albrigo, 2013a; Tang, 2017; Tang and Lovatt, 2019). However, literature on the subject is relatively scarce and concrete conclusions are yet to be made.

2.8. Literature cited

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2.9. Appendix

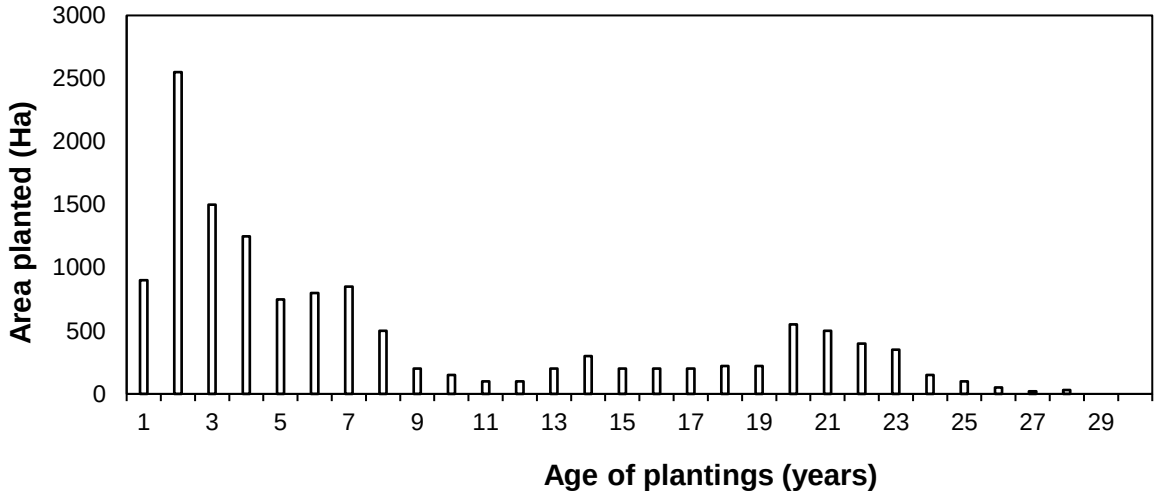

Fig. 2.1. Age distribution (years) of South African lemon orchards. Adapted from CGA (2019).

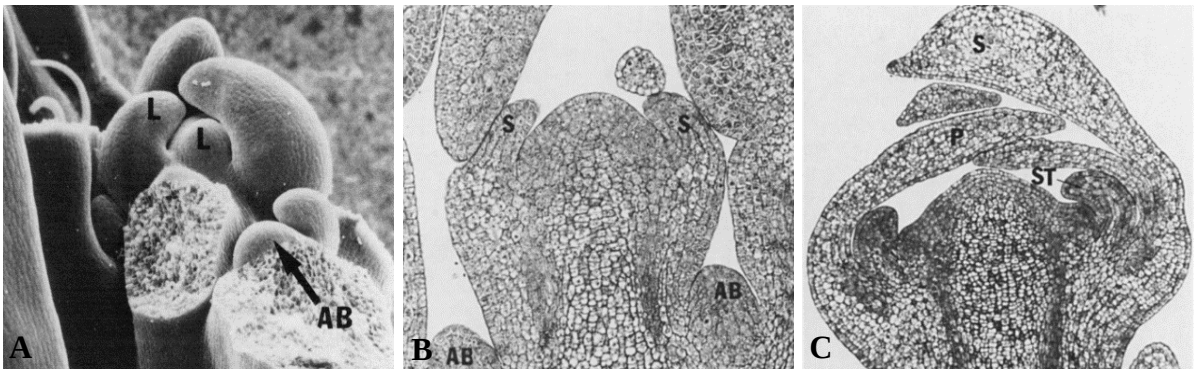

Fig. 2.2. Different stages of flower development: A) vegetative apex with leaves (L) and axillary bud (AB); B) Beginning of sepal (S) initiation, with bud primordia in axils of the foliar leaves; C) Floral bud starting to differentiate, showing sepals, petals (P) and stamen (ST). Adapted from Lord and Eckard (1985, 1987).

Table 2.1. List of relevant studies evaluating the effect of winter foliar Gibberellin (GA) sprays on spring flowering of citrus.

Species	Cultivar	Treatment	Timing	Region	Response vs. control	Reference
Citrus sinensis L. Osbeck	'Shamouti' sweet orange	200 mg·L^{-1} GA_3	Four fortnightly sprays in midwinter	Rehovot, Israel	0.5 Flowers per tree vs. 80 flowers per control tree.	Monselise and Halevy (1964)
C. sinensis L. Osbeck	'Washington navel' sweet orange	200 mg·L^{-1} GA_3	Two fortnightly sprays in midwinter	Greenhouse conditions	93% decrease in spring flowering	Moss (1970)
C. sinensis L. Osbeck	'Navelate' sweet orange	100 mg·L^{-1} GA_3	Five fortnightly sprays in midwinter	Valencia, Spain	50% decrease in spring flowering	Guardiola et al. (1977)
C. unshiu Marc.	'Satsuma' mandarins	25 mg·L^{-1} GA_3	A single spray in midwinter (November NH)	Valencia, Spain	300% decrease in spring flowering	Guardiola et al. (1982)
C. sinensis L. Osbeck	'Salustiana' sweet orange	40 mg·L^{-1} GA_3	A single spray in midwinter (December NH)	Valencia, Spain	71% decrease in spring flowering	Muñoz-Fambuena et al. (2012)
C. limon	'Eureka' lemon	40 mg·L^{-1} GA_3	Two sprays in midwinter (November NH)	North-Eastern Tunisia	25% decrease in spring flowering	Khelil et al. (2013)
C. reticulata	'Nadorcott' mandarin	40 mg·L^{-1} GA_3	Two sprays at the start of winter (May/June SH)	Citrusdal, South Africa	93% decrease in spring flowering	Stander (2018)

Table 2.2. List of relevant studies evaluating the effect of water-deficit stress on flowering of citrus species.

Species	Cultivar	WD stress intensity	Duration	Timing	Region	Response vs control	Reference
Citrus latifolia Tan.	'Tahiti' lime	-2 MPa PDXPP[a]	2 weeks	Greenhouse conditions	Not applicable (NA)	Increase of 247 flowers per tree	Southwick and Davenport (1986)
C. limon Burm.	'Femminello comune' lemon	-3.08 MPa PDXPP	10 weeks	Summer (May NH)	Palermo, Italy	Double the amount of flowers/m^3	Barbera and Carimi, 1988
C. limon	'Frost Lisbon' lemon	< -3 MPa PDXPP	30 days	Summer (June NH)	California, USA	Increase of 39 flowers per tree	Lovatt et al., 1988a, b
C. limon	'Frost Lisbon' lemon	-2 – -3 MPa PDXPP	-3 MPa for 20 days, then -2 MPa for 30 days	Summer (June NH)	California, USA	Increase of 507 flowers per tree	Hake, 1995
C. sinensis	'Washington Navel' sweet orange	-2 MPa SWP[b]	60 days	Greenhouse conditions	NA	Increase of 3 flowers per shoot	Chica and Albrigo, 2013a
C. sinensis	'Washington Navel' sweet orange	-2.8 MPa SWP	8 weeks	Greenhouse conditions	NA	Increase of 40 flowers per tree	Tang (2017)

[a] Pre-dawn xylem pressure potential

[b] Stem water potential

CHAPTER 3

The effect of gibberellic acid on lemon (*Citrus limon*) reproductive development

ABSTRACT

Gibberellins (GAs) are known to inhibit flowering in *Citrus* spp. when applied before sepal formation, however, it is not clear if floral induction or floral differentiation is affected in the inhibitive process. Furthermore, the effects of GAs on the expression of genes involved in citrus flowering are yet to be determined, although their effects on the *FLOWERING LOCUS T* (*FT*) gene expression is very likely. The aim of this study was to inhibit spring flowering of lemon trees through gibberellic acid (GA_3) foliar spray applications, as well as quantifying the expression of known citrus floral genes involved in this process. This was achieved by the application of two GA_3 foliar spray applications to lemon and lime trees in early winter. Foliar application of GA_3 in winter resulted in a reduction in spring flowering with the lowest concentration of GA_3 treatment, viz., 10 $mg \cdot L^{-1}$, causing floral inhibition to the same extent as 20 and 40 $mg \cdot L^{-1}$ GA_3 treatments. In most cases, GA_3 treatments resulted in a significant increase in new spring vegetative shoot growth. Expression levels of *FT* and *APETALA1* were significantly lower in buds of trees that received GA_3 applications, whereas *AGAMOUS* was not sufficiently expressed to quantify. It may, therefore, be concluded that the effect of GA_3 on lemon spring flowering is due to inhibition of floral induction, most likely as a result of the down-regulation of *FT* expression which derails the rest of the floral cascade.

ADDITIONAL INDEX WORDS: *APETALA1*, floral differentiation, floral induction, *FLOWERING LOCUS T*, gibberellic acid, uniconazole

3.1 Introduction

Gibberellins (GAs) are known to initiate growth and stimulate cell division and cell enlargement in plant organs (Iglesias et al., 2007). In addition, GAs can induce reproductive development in a large number of annual plant species, specifically long-day and/or cold requiring angiosperms (Goldschmidt et al., 1997). In perennial fruit crops such as *Citrus* spp., however, GAs inhibit flowering (Lord and Eckard, 1987). After initial reports from Monselise

and Halevy (1964), several additional studies followed which emphasised the effectiveness of GAs in inhibition of citrus flowering, especially gibberellic acid (GA_3) (Goldschmidt and Monselise, 1977; Lord and Eckard, 1987; Moss, 1970). Gibberellins are known to shift the balance of a citrus tree from a reproductive to a vegetative phase, and to increase the proportion of leafy inflorescences as opposed to leafless inflorescences (Moss, 1970; Spiegel-Roy and Goldschmidt, 1996). Therefore, it is applied frequently for control of alternate bearing in commercial citrus production (Guardiola et al., 1977).

Lemon [*Citrus limon* (L.) Burm. f.] trees that are grown in a subtropical climate start reproductive development during the cool temperatures of winter and flower profusely thereafter during spring, resulting in a crop that is typically harvested during the following winter (Davies and Albrigo, 1994; Hake, 1995). Goldschmidt et al. (1997) proposed that a sustained period of low temperature results in a reduction in the endogenous concentration of GAs in citrus tree organs, which triggers floral formation.

Gibberellins are produced in various tree organs, of which actively growing rootlets are a major source (Carr et al., 1964; Goldschmidt et al., 1997; Spiegel-Roy and Goldschmidt, 1996). Therefore, the restriction of root growth at the onset of winter due to low soil temperature and the subsequent decrease in GA translocation to the top parts of the tree have been proposed to be involved in the stimulation of floral development (Spiegel-Roy and Goldschmidt, 1996). The use of GA biosynthesis inhibitors supports the involvement of GA in citrus flowering. Gibberellin biosynthesis inhibitors such as the triazoles uniconazole and paclobutrazol, reduce endogenous GA concentration and, if applied correctly, support reproductive development by decreasing the inhibitory effect of GAs (El-Otmani et al., 2000; Monselise and Halevy, 1964; Monselise et al., 1966).

The physiological mechanism by which GAs inhibits flowering in fruit trees is still unclear. Early studies proposed that GAs merely increases vegetative growth which competes with reproductive growth for assimilates and subsequently has an indirect effect on flowering (Goldschmidt and Monselise, 1972; Sachs et al., 1967). Later studies have, however, pointed to a more direct effect of GA on flowering, as GA applications were found to decrease flowering without affecting vegetative growth (Goldshmidt and Monselise, 1972). Goldschmidt et al. (1997) hypothesised that the relationship between flowering and GA could be attributed to inflorescence stem elongation. Flowering in herbaceous angiosperms is related

to elongation of the inflorescence axis (Goldschmidt et al., 1997), whereas, in citrus, flowering occurs on short bearing units and there is a negative linear relationship between flowering intensity and stem elongation (Goldschmidt and Monselise, 1972). Therefore, the authors put forth the idea that GAs are always responsible for stem elongation, whereas it has an opposite effect on flowering between herbaceous angiosperms and woody perennials.

Winter GA_3 foliar applications have been found to effectively inhibit spring flowering of various citrus cultivars, including lemon (Guardiola et al., 1977; 1982; Monselise and Halevy, 1964). It is generally accepted that the presence of GA_3 throughout the cold winter months, coinciding with the whole period of flower formation, inhibits spring flowering as long as it is applied before sepal formation (Goldschmidt et al., 1997; Lord and Eckard, 1987). However, it is not clear which stage of floral formation is affected (Goldschmidt et al., 1997). Previous authors have found GA_3 applications to be especially effective in inhibiting flowering if applied during the floral induction period, i.e. in early to mid-winter (Guardiola et al., 1977; Khelil et al., 2013). However, some results indicate that later GA_3 applications, correlating with the floral differentiation period, are also effective (Guardiola et al., 1982; Monselise and Halevy, 1964). Applications in early winter most likely reduce spring flowering by inhibiting floral induction, while late-winter applications when bud differentiation has already commenced, are thought to inhibit bud sprouting (Goldberg-Moeller et al., 2013). Recent studies have focused on identifying the effect of GA_3 applications on the expression of citrus flowering genes to shed light on the specific flower developmental process affected (Goldberg-Moeller et al., 2013; Muñoz-Fambuena et al., 2012; Tang and Lovatt, 2019).

In the model plant *Arabidopsis thaliana*, *FLOWERING LOCUS T* (*FT*) is known to play a central role in triggering the flowering cascade, specifically by integrating different floral pathways and stimulating the expression of floral meristem identity genes, including *APETALA1* (*AP1*) (Abe et al., 2005; Horvath, 2009; Parcy, 2005; Taiz et al., 2018). The expression of these floral meristem identity genes lead to the up-regulation of floral organ identity genes, including *AGAMOUS* (*AG*) (Taiz et al., 2018). It has been proven that homologs of these genes function similarly in citrus and other woody species (Endo et al., 2005; Nishikawa et al., 2009; Pillitteri et al., 2004; Tan and Swain, 2007; Tang and Lovatt, 2019).

Insights on the effects of GA_3 foliar applications on the expression of citrus floral genes are restricted to only a handful of studies. A study by Muñoz-Fambuena et al. (2012) showed that

GA_3 applications down-regulate the expression of *FT* in buds of sweet orange [*C. sinensis* (L.) Osbeck] during the floral induction period, indicating that GA_3 directly inhibits floral induction. Similarly, Goldberg-Moeller et al. (2013) found that GA_3 sprays reduced flowering of 'Orri' mandarin (*C. reticulata* Blanco) by inhibiting the expression of *FT* during floral induction. In contrast, a recent study by Tang and Lovatt (2019) found that GA inhibits floral development in 'Washington navel' sweet orange by affecting the expression of *AP1*. In their study, they found that four to six weekly applications of GA_3 to 'Washington navel' sweet orange trees at low temperatures had no effect on *FT* expression. However, the expression of *AP1* was significantly reduced. Tang (2017) also found that *AP1* was down-regulated when GA_3 was applied to trees under water-deficit stress, while the expression of *FT* was not affected.

The aim of this study was to confirm the efficacy of foliar application of GA_3 during the floral induction period in inhibiting spring flowering of lemon trees. Additionally, this study attempted to provide insight into the effect of foliar GA_3 applications on the expression of important lemon flowering genes to better understand the molecular mechanisms involved.

3.2. Materials and Methods

3.2.1. Plant material and experimental sites

Three experimental sites were selected in the Western Cape province of South Africa. These areas experience a Mediterranean climate.

'Lisbon' lemon, De Doorns

The experiment was conducted during the 2018/2019 season in De Doorns (33°51'S 19°51'E), South Africa, on non-bearing, four-year-old 'Lisbon' lemon trees budded onto 'Carrizzo' citrange (*C. sinensis* × *Poncirus trifoliata* L. Raf.) rootstock. The orchard was planted at a spacing of 5 m between rows and 2 m between trees in a North-to-South row-direction.

'Eureka seedless' lemon, Stellenbosch

The experiment was conducted during the 2018/2019 and 2019/2020 seasons on the Welgevallen experimental farm in Stellenbosch (33°95'S 18°88'E), South Africa, on non-bearing two-year-old 'Eureka Seedless' lemon trees budded onto 'Rough lemon' [*C. jambhiri*

(Lush.)] rootstock. The orchard was planted at a spacing of 5 m between rows and 3 m between trees in a North-to-South row-direction.

'Bearss lime', Citrusdal

The experiment was conducted during the 2019/2020 season in Citrusdal (32°44'S 19°03'E), South Africa, on eight-year-old 'Bearss' lime (*C. latifolia* Tan) trees budded onto 'Rough lemon' rootstock. The orchard was planted at a spacing of 6 m between rows and 3 m between trees in an East-to-West row-direction.

3.2.2. Treatments and experimental design

The experiment was set up in a randomised complete block design with seven replicates (trees) per treatment (n=7) in De Doorns and Stellenbosch, and six replicates (trees) per treatment (n=6) in Citrusdal. Replicate trees were selected for uniformity in trunk circumference above the bud union, canopy density, and tree size prior to treatment application. Buffer trees were located between treatment replicates within the treatment rows. Standard commercial orchard practices that were aimed at obtaining export quality fruit were followed in all orchards. A gibberellic acid (GA_3) [Progibb® 40% soluble granule (SG) formulation; Philagro SA (Pty) Ltd, Somerset West, South Africa, 400 $g \cdot kg^{-1}$ GA_3] foliar spray treatment was applied twice to trees in May, two weeks apart, to ensure high endogenous GA concentrations during the floral induction and –initiation period. Gibberellic acid was shown to inhibit 'Nadorcott' mandarin flowering in the Western Cape province of South Africa when applied in May (Stander, 2018). Temperature was monitored using loggers (Tinytag®, Plus 2, Gemini Data Loggers, Chichester, UK) to ensure floral inductive conditions. Three GA_3 concentrations were applied as separate treatments, viz., 10, 20 and 40 $mg \cdot L^{-1}$. Treatments were applied with a motorised backpack sprayer (STIHL, Pietermaritzburg, South Africa) in the first and third weeks of May, until the point of runoff.

3.2.3. Data collection and evaluations

3.2.3.1. Gene expression

Sample collection

The first five apical buds from the terminal position of five non-bearing shoots per tree were collected from the Stellenbosch site, one day prior to treatment and two weeks after application of the second 40 $mg \cdot L^{-1}$ GA_3 treatment for both the treatment and control samples. Collected buds were promptly placed in a plastic bag in an ice-filled cooler box for immediate transport

to the laboratory for extraction. Bud samples were finely ground in liquid nitrogen using mortar and pestle, and immediately stored at -80 °C until further analysis.

Total RNA extraction

Total RNA was extracted from the finely ground bud tissue using a Quick-RNA™ Miniprep kit (Zymo Research, Irvine, CA). The quality and quantity of RNA was analysed with a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). To degrade any DNA, samples were treated with RQ1 RNase-free DNase (Promega, Madison, USA) according to the manufacturer's instructions. After the addition of 450 µl of 10 mM Tris-HCl (pH 8.5) to the DNase treated sample, an acidic phenol:chloroform-isoamyl alcohol (5:1) extraction was carried out followed by an ethanol and sodium acetate precipitation [2.5 volumes of 100% ethanol and 0.1 volumes of 3 M Sodium acetate (pH 5.2)]. A wash step was performed with 70% ethanol and pellets were dried and re-suspended in 30 µl Milli-Q H_2O. Finally, spectrophotometric analysis and agarose gel electrophoresis were carried out to evaluate the integrity and purity of DNase-treated samples.

cDNA synthesis

Complementary DNA (cDNA) was synthesised from 500 ng of total RNA using the ProtoScript™ First Strand cDNA Synthesis Kit with the Random Primer Mix (New England Biolabs, Beverly, MA, USA) in a final volume of 20 µl. Ten microliters of each cDNA sample were pooled and a five-fold dilution series prepared to be used for qPCR standard curves. The remaining cDNA was diluted (1:24) to create the unknown samples for quantitation before they were stored at -20 °C.

Genes of interest

The citrus homologs of the flowering genes *FT*, *AP1* and *AG* were selected to be quantified in this study as their expression coincides with distinct developmental phases in the flowering cascade, namely induction, initiation and differentiation, respectively (Taiz et al., 2018). Primer sequences for all target genes are displayed in Table 3.1. Additionally, the expression of the citrus homologs of *FT* and *AP1* is altered upon GA_3 treatments (Goldberg-Moeller et al., 2013; Muñoz-Fambuena et al., 2011; Tang and Lovatt, 2019).

Reference gene selection and stability test

The β-Actin (*ACT*) and glyceraldehyde-3-phosphate dehydrogenase C2 (*GAPC2*) genes were selected as reference genes due to their stability in qPCR analysis across citrus genotypes (Mafra et al., 2012; Yan et al., 2012). Primer sequences for all reference genes are displayed in Table 3.1. The stability of reference gene expression was calculated from the quantitation cycle (Cq) data for all 30 samples (three samples per 10 plants) using Bestkeeper (Pfaffl et al., 2004), an excel-based application.

qPCR

qPCRs were performed using the Rotor-gene Q thermal cycler (Qiagen, Venlo, Netherlands) and the Luna® Universal qPCR Master Mix kit (NEB, Ipswich, MA, USA). Reactions were made up to 10 µl, containing 5 µl 2 × Luna® Universal qPCR Master Mix, 2 µl Milli-Q H_2O, 0.125 µl forward and reverse primers (Table 3.1), as well as 2 µl, diluted cDNA. The same cDNA dilution series was used for the five primer-specific standard curves (three genes of interest and two reference genes). Quantitation was achieved by screening the same 1:24 dilution sample with the five primer sets. No-template controls and no-reverse transcription controls were included in all runs. Reactions were performed in triplicate in Qiagen Rotor-gene Q 0.1 mL tube-and-cap strips. Each reaction was run at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Melt-curve analyses were performed at temperatures ranging between 60 and 95 °C, with a 1 °C increase every 5 s to identify primer-dimers and non-specific amplification.

Data analysis

For both treatments on each sampling date the mean of five biological replicates (trees) was used to quantify gene expression. Each biological replicate was the mean of three qPCR technical replicates. Rotor-gene Q software 2.3.1 (Qiagen) was utilised to calculate PCR efficiency, Cq-, and quantitation values for all targets. These calculations were performed using the slope of the standard curve derived from the pooled five-fold dilution series for each gene. The second dilution point (25X) of the five-fold dilution series for each gene was included in all runs to compensate for inter-assay variability. The relative concentration ratio (CR) for each sample was calculated with the geometric mean of the triplicate reactions and normalised using a reference gene index, which is the geometric mean of the two reference genes. Harbin (https://rbester.shinyapps.io/Harbin/), a web-based software application developed by Bester et al. (2017) was utilised to simplify the analyses and perform the calculations.

3.2.3.2. Bud development

Ten non-fruiting, purely vegetative shoots with a length of 10-15 cm were selected and tagged on each replicate tree on the day of the first foliar spray treatment in May, before treatment application. During flowering in spring, the number of inflorescences and new vegetative shoots that sprouted from each tagged shoot was counted at weekly intervals throughout the spring flowering period. The average amount of flowers and new vegetative shoots per branch per treatment was determined for statistical analysis.

3.2.4. Statistical analysis

Analyses of variance (ANOVA) were performed using STATISTICA [Dell Inc. 2015, Dell Statistica (Data analysis software system) version 13. software.dell.com]. Repeated measures analyses of variance (RANOVA) were used to test for treatment effects on the number of inflorescences and vegetative shoots per tree over time (treatment × time). Mean separations were carried out using the least significance difference test (Fisher's LSD). A *P* value significance threshold of 0.05 was selected in all instances.

3.3. Results

3.3.1. Ambient temperature

Minimum ambient temperatures measured at Welgevallen, Stellenbosch during the sampling period in the 2017/2018 season frequently dropped beneath 10 °C (Fig. 3.1).

3.3.2. Gene expression

Reference gene expression

BestKeeper was utilised to validate the expression stability of citrus reference genes used in this study, *Actin* and *GAPC2*. The descriptive statistics for the respective reference gene and the BestKeeper indices are presented in Table 3.2. Expression stability can be analysed using variations in Cq, quantified by standard deviations (SD) and coefficient of variances. Standard deviations higher than 1 are an indication of reference gene inconsistency (Pfaffl et al., 2004). Both reference genes used in this study were shown to be sufficiently stable in the samples analysed, with Cq standard deviations (SD) lower than 1 (Table 3.2).

Relative quantitation

Transcripts of *FT* and *AP1* were detected in buds from shoots sampled on both sampling dates in both the control and GA_3 treatments (Fig. 3.2). However, on both sampling dates, the transcript level of *AG* was too low to continue qPCR quantitation (data not shown). In buds of trees that were sampled before treatment application, the level of relative expression of both *FT* and *AP1* were similar to the control as expected (Fig. 3.2). However, after GA_3 foliar spray treatment, buds showed significantly lower levels of expression for both *FT* ($P = 0.011$) and *AP1* ($P = 0.049$) compared to the control (Fig. 3.2).

3.3.3. Bud development

'Lisbon' lemon, De Doorns

In the 2018/2019 season, two winter foliar spray applications of 10 $mg \cdot L^{-1}$ GA_3 each in May caused a significant increase in new vegetative shoot growth in spring, with double the amount of new vegetative shoots that developed per branch compared with control trees (Table 3.3). However, 20 and 40 $mg \cdot L^{-1}$ GA_3 foliar sprays, respectively, had no effects on new vegetative shoot growth compared with the control and 10 $mg \cdot L^{-1}$ GA_3 foliar spray treatments (Table 3.3).

All the winter GA_3 foliar sprays resulted in a highly significant ($P < 0.0001$) reduction of spring flowering compared with the control, with a minimum spring-flowering reduction of 64% (Table 3.3). There were no significant differences in spring-flowering response between the different GA_3 foliar spray treatments (Table 3.3).

'Eureka seedless' lemon, Stellenbosch

In the 2018/2019 season, 20 and 40 $mg \cdot L^{-1}$ GA_3 foliar sprays resulted in a significant increase in the number of new vegetative shoots compared with the control and 10 $mg \cdot L^{-1}$ GA_3 foliar spray treatments (Table 3.4). The two 10 ppm GA_3 foliar sprays did not affect the number of new vegetative shoots compared with the control (Table 3.4).

Spring flowering was significantly reduced ($P < 0.0001$) by all the different winter GA_3 treatments (Table 3.4). Trees treated with GA_3 had a minimum of 77% fewer flowers per branch in spring compared with the control (Table 3.4). However, there were no significant differences in the spring flowering response between the respective GA_3 foliar spray treatments (Table 3.4).

In the 2019/2020 season, GA_3 foliar sprays did not affect the extent of vegetative growth during spring compared with the control (Table 3.4). All the winter GA_3 foliar sprays caused a significant decrease in spring flowering, with a minimum of 40% fewer flowers per branch compared to the control (Table 3.4). There were no significant differences in spring flowering between the respective GA_3 foliar spray treatments.

'Bearss' lime, Citrusdal

In the 2019/2020 season, 40 $mg \cdot L^{-1}$ GA_3 foliar sprays resulted in a significant increase in the number of new vegetative shoots per branch compared with the control and the 10 and 20 $mg \cdot L^{-1}$ GA_3 foliar spray treatments (Table 3.5). The 10 and 20 $mg \cdot L^{-1}$ GA_3 foliar sprays did not affect new vegetative growth compared with the control (Table 3.5).

All the GA_3 foliar sprays resulted in a significant ($P < 0.0001$) reduction in spring flowering compared with the control (Table 3.5). The 20 and 40 $mg \cdot L^{-1}$ GA_3 foliar sprays inhibited spring flowering to a significantly greater extent than the 10 $mg \cdot L^{-1}$ spray treatments (Table 3.5). There were no significant differences in spring flowering between the 20 and 40 $mg \cdot L^{-1}$ GA_3 foliar spray treatments.

3.4. Discussion

In this study, two foliar applications of GA_3 to trees in early winter (May), which coincides with the floral induction period according to previous studies (Nishikawa et al., 2007; Stander, 2018; Valiente and Albrigo, 2004) successfully inhibited lemon and lime flowering. Additionally, the expression of the *FT* gene in buds of lemon trees in early winter was inhibited by a single GA_3 application.

Reproductive development of lemon trees and other *Citrus* spp. commences during a period of low temperatures in winter, followed by anthesis in spring (Davies and Albrigo, 1994; Hake, 1995). The inhibitory effect of winter application of GAs on citrus flowering is well documented (Goldberg-Moeller et al., 2013; Khelil et al., 2013; Lord and Eckard, 1987; Muñoz-Fambuena et al., 2012), but the floral development period most sensitive to GA_3 applications is yet to be comprehensibly proven. Since the expression of the *FT* gene has been proven to be integral to the commencement of floral induction in citrus, these results suggest that the floral development process is sensitive to GA_3 application specifically during the floral

induction period. This study shows similar down-regulation of *FT* to studies by Goldberg-Moeller et al. (2013) and Muñoz-Fambuena et al. (2012). The dosages chosen (10, 20 and 40 $mg \cdot L^{-1}$) were based on similar studies proving that foliar sprays of 20 $mg \cdot L^{-1}$ were effective in inhibiting flowering during the floral formation period (Khelil et al., 2013; Monselise, 1979). In this study, the 10 $mg \cdot L^{-1}$ dosage consistently inhibited flowering and to the same extent as the 40 $mg \cdot L^{-1}$ dosage, proving that GA_3 is highly active at low concentration.

The expression of the floral meristem identity gene, *AP1*, which is associated with floral development later in the flowering cascade, was also significantly reduced by early winter foliar GA_3 application. This coincides with results by Tang and Lovatt (2019) on 'Washington' navel orange, although they found no reduction in the relative expression of *FT* during the whole floral development process. The effect of GA_3 on *FT* expression in this study could be due to species differences. Therefore, it is not clear whether *AP1* is directly affected by GA_3 applications or if it is simply down-regulated as a result of reduced expression of floral genes involved earlier in the flowering cascade, such as *FT*.

Increased spring vegetative shoot sprouting was observed in lemon and lime trees treated with winter foliar GA_3 applications. This increased shoot growth was not consistently shown across all treatment concentrations, yet at most trial sites there was at least one GA_3 dosage that resulted in more spring vegetative shoot growth compared to control trees. These results concur with studies by Guardiola et al. (1982) and Muñoz-Fambuena et al. (2012).

Fruit load has been known to reduce return bloom in various citrus cultivars (Iglesias et al., 2007; Muñoz-Fambuena et al., 2011) and the presence of fruit has been proven to alter floral gene expression in buds of 'Moncada' mandarin (Muñoz-Fambuena et al., 2011). Therefore, young non-bearing orchards were used in this study to nullify any effect of fruit load on flowering. The observed increase in vegetative shoot growth, coupled with the inhibition of flowering, can be of commercial importance to producers aiming to enhance vegetative shoot growth and decrease reproductive growth of newly established orchards. Flower-inhibiting gibberellin sprays have predominately been administered in commercial citriculture for the control of biennial bearing, where winter applications preceding an expected "on" year are used to decrease return bloom, similar to the effect of a high crop load during floral formation in an "on" year (El-Otmani et al., 2000; Monselise, 1979; Moss, 1970). The results of the current

study further confirm the efficacy of GA_3 sprays for the control of alternate bearing in citrus production.

For gene expression quantitation in this study, apically located buds were collected at the onset of winter (early May). During the sampling period, average day and night temperatures fluctuated between the known optimum ranges of floral induction for citrus; 10 to 18 °C and 8 to 13 °C, respectively (Lovatt et al., 1992; Tang and Lovatt, 2019; Nishikawa et al., 2007; Southwick and Davenport, 1986; Valiente and Albrigo, 2004). Therefore, it can be assumed that the process of floral induction was initiated at the time of sampling. Goldberg-Moeller et al. (2013) showed that the relative expression of *FT* was higher in buds than in leaves of 'Orri' mandarin trees. Furthermore, Nishikawa et al. (2007) found a stronger correlation between flowering and mRNA levels in shoots than in leaves. Consequently, apical buds were analysed for gene expression in the current study.

In conclusion, this study proves that winter GA_3 foliar sprays can decrease lemon spring flowering by inhibiting floral induction. This inhibition has been proven to be, at least in part, due to the down-regulation of *FT* and *AP1* during floral induction. Additionally, an increase in vegetative growth was observed.

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Table 3.1. Forward and reverse primer sequences for the citrus target and reference genes used in the quantitative real-time PCR (qPCR) assays.

Gene abbreviation	Annotation	Forward primer (5' to 3') Reverse primer (5' to 3')	Amplicon length (bp)	Primer concentration (µM)	Mean efficiency (%)	Reference
FT	Flowering locus T	CCGCGTTGTTGGTGATGTTCTTGA ATTTCAGCCCTAGGCTGGTTCAGA	132	0.25	80	Tang and Lovatt (2019)
AP1	Apetala 1	ACCGCTCTCAAACACATCAG GCAGCCTCTCTCTCTCC	137	0.25	77.5	Tang and Lovatt (2019)
AG	Agamous	GGGAAGTTGACTTGCACAACAGCA TAGCTCCGGGAATCAAATGGCTGA	142	0.25	NA[z]	Tang and Lovatt (2019)
ACT	Actin	TCACAGCACTTGCTCCAAGCAG TGCTGGAAGGTGCTGAGGGA	130	0.25	90	Tang and Lovatt (2019)
GAPC2	Glyceraldehyde-3-phosphate dehydrogenase C2	TCTTGCCTGCTTTGAATGGA TGTGAGGTCAACCACTGCGACAT	80	0.25	79	Mafra et al. (2012)

[z] Expression value below the threshold for detection (quantitation cycle [Cq] in qPCR > 35).

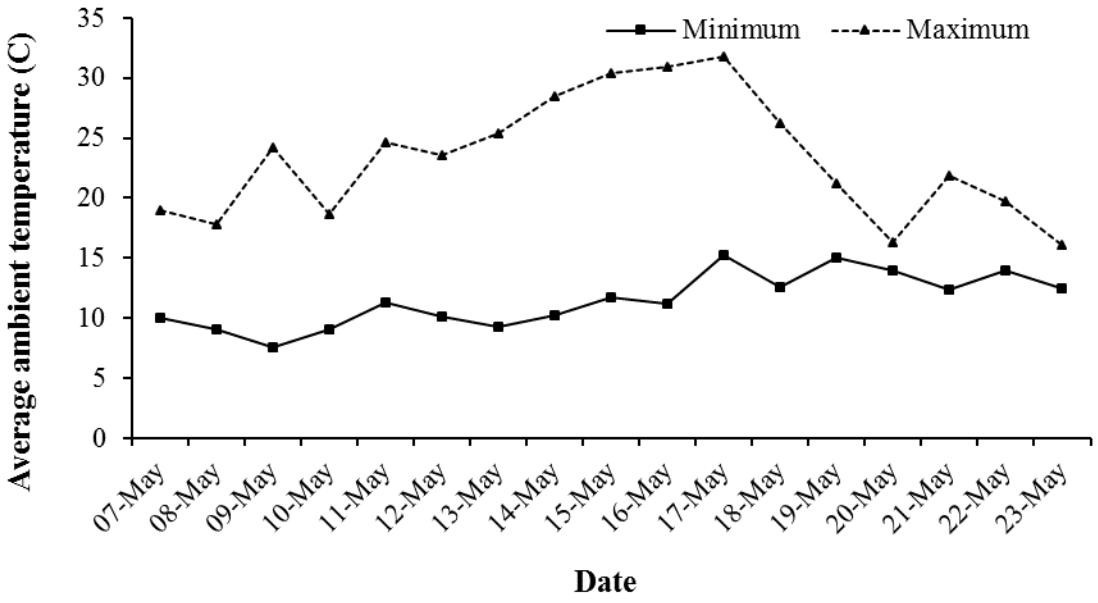

Fig. 3.1. Maximum and minimum ambient temperatures (ºC) during the sampling period (2017/2018 season) at Welgevallen experimental farm, Stellenbosch, South Africa. Temperatures were measured using an air temperature logger (TinyTag®, Plus 2, Gemini Data Loggers, Chichester, UK).

Table 3.2. BestKeeper descriptive statistics of candidate reference genes, the pairwise correlation between reference genes and pairwise comparison between each reference gene and the calculated BestKeeper index.

	Candidate reference genes	
	Actin	*GAPC2*
Number of samples	30	30
Geometric mean (Cq)	21.89	18.52
Arithmetic mean (Cq)	21.9	18.53
Minimum (Cq)	21.16	17.91
Maximum (Cq)	23.24	19.52
Standard deviation (±Cq)	0.45	0.41
Coefficient of variation (%Cq)	2.05	2.19
Pearson correlation coefficient (r) analysis vs		
GAPC2	0.907	
P value	0.001	
BestKeeper index vs correlation coefficient (r)	0.976	0.977
P value	0.001	0.001

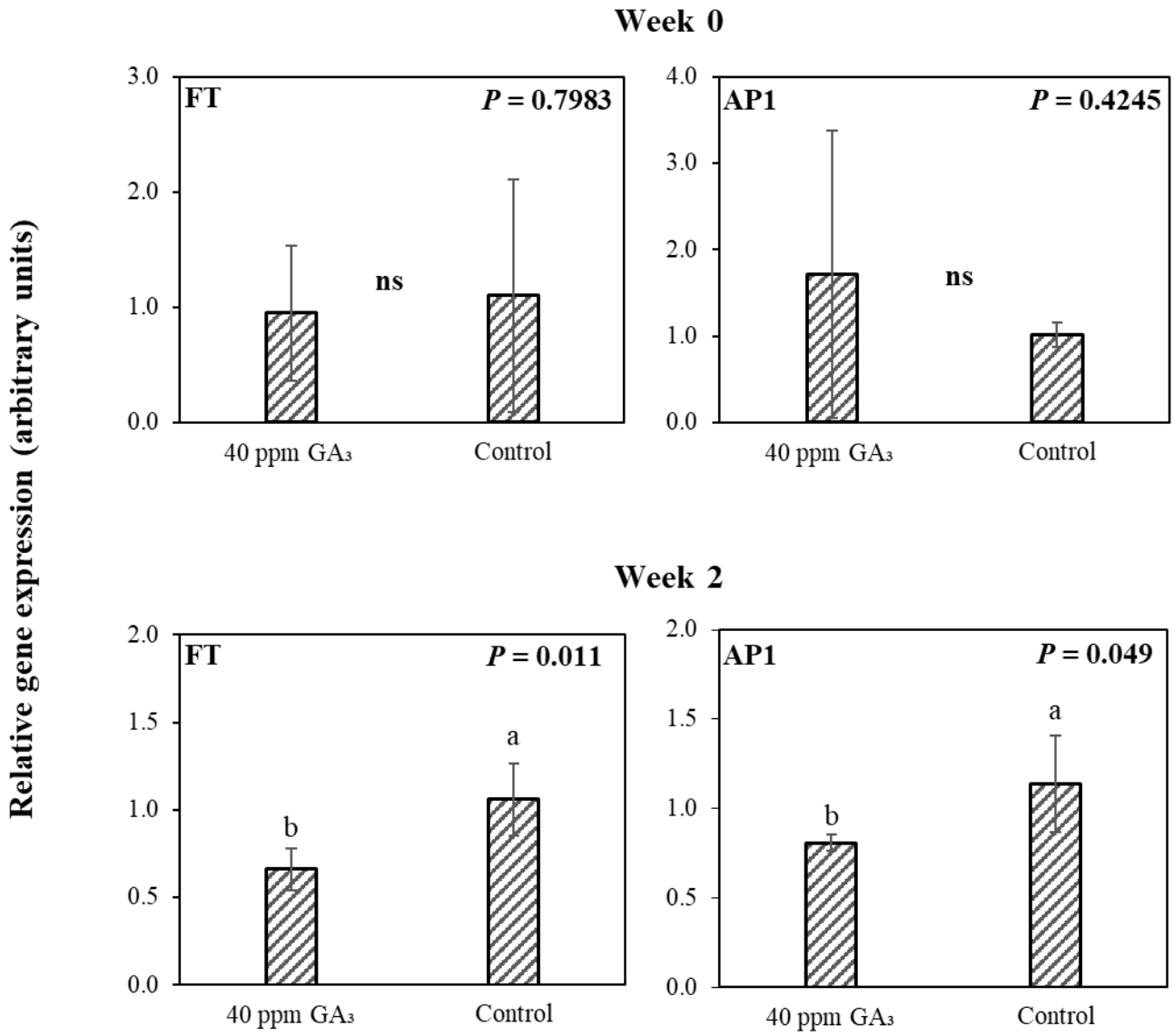

Fig. 3.2. Relative expression of *FT* and *AP1* in buds of 'Eureka Seedless' lemon trees receiving winter GA_3 foliar spray treatments and untreated control trees at the Welgevallen Experimental farm, Stellenbosch, Western Cape, South Africa in 2018. Week 0 and week 2 indicate sampling before treatment applications, and two weeks after the day of the first treatment, respectively. Vertical bars represent mean concentration ratios of five biological replicates with standard deviation bars. Different letters indicate statistically significant differences ($P < 0.05$) between the control and treatment. ns = No significant difference.

Table 3.3. The effects of different concentrations of winter foliar gibberellic acid (GA_3) sprays on spring vegetative shoot growth and flowering response of 'Lisbon' lemon trees during the 2018/2019 season, in De Doorns, South Africa.

Treatment	Veg. shoots per branch	Flowers per branch
Control	0.3b[y]	11.8a
2 x 10 mg·L^{-1} GA_3[z]	0.7a	4.3b
2 x 20 mg·L^{-1} GA_3	0.4b	3.0b
2 x 40 mg·L^{-1} GA_3	0.4b	3.1b
P value	0.0130	< 0.0001

[z] Progibb® 40% soluble granule (SG) formulation [Philagro SA (Pty) Ltd, Somerset West, South Africa, 400 g·kg^{-1} GA_3].

[y] Means with a different letter within a column differ significantly at the 5% level (least significant difference).

Table 3.4. The effects of different concentrations of winter foliar gibberellic acid (GA_3) sprays on spring vegetative shoot growth and flowering response of 'Eureka Seedless' lemon trees at Welgevallen experimental farm, Stellenbosch, South Africa.

Treatment	Veg. shoots per branch		Flowers per branch	
	2018/19	2019/20	2018/19	2019/20
Control	0.8c[y]	0.4[ns]	3.2a	4.8a
2 x 10 mg·L^{-1} GA_3[z]	1.2bc	0.6	0.7b	2.8b
2 x 20 mg·L^{-1} GA_3	1.6a	0.7	0.3b	2.3b
2 x 40 mg·L^{-1} GA_3	1.3b	0.7	0.3b	2.9b
P value	0.0004	0.2186	< 0.0001	0.0001

[z] Progibb® 40% soluble granule (SG) formulation [Philagro SA (Pty) Ltd, Somerset West, South Africa, 400 g·kg^{-1} GA_3].

[y] Means with a different letter within a column differ significantly at the 5% level (least significant difference).

[ns] No significant differences.

Table 3.5. The effects of different concentrations of winter foliar gibberellic acid (GA_3) sprays on spring vegetative shoot growth and flowering response of 'Bearss' lime trees during the 2019/2020 season, in Citrusdal, South Africa.

Treatment	Veg. shoots per shoot	Flowers per shoot
Control	0.2b[y]	5.2a
2 x 10 mg·L^{-1} GA_3[z]	0.2b	4.0b
2 x 20 mg·L^{-1} GA_3	0.2b	2.9c
2 x 40 mg·L^{-1} GA_3	0.5a	2.7c
P value	0.0396	< 0.0001

[z] Progibb® 40% soluble granule (SG) formulation [Philagro SA (Pty) Ltd, Somerset West, South Africa, 400 g·kg^{-1} GA_3].

[y] Means with a different letter within a column differ significantly at the 5% level (least significant difference)

CHAPTER 4

Attempts to adapt the *forzatura* technique to lemon production in the Western Cape Province of South Africa

ABSTRACT

Increased lemon production has brought about fears of a possible future oversupply of winter lemons (*Citrus limon*) in South Africa. These fears could be addressed by shifting the lemon supply peak to a period of lower competition and higher demand, i.e., summer. Sicilian citriculturists have applied the *forzatura* technique for centuries. This technique consists of withholding irrigation during the warm and dry summer months to induce controlled water-deficit (WD) stress. Once sufficiently stressed, trees are re-irrigated and subsequently flower in late summer or autumn to produce a summer crop called *Verdelli* lemons. The effects of the *forzatura* technique on the expression of citrus flowering genes have not been researched extensively and could provide valuable insight into the mechanism of reproductive development. This study attempted to adapt the *forzatura* technique under South African conditions and to evaluate the effect of controlled WD stress on the expression of key citrus flowering genes. In trials where controlled WD stress reduced stem-water potential to below -2.5 MPa, a significant flowering reaction was obtained three to six weeks upon re-irrigation. Supplementary nitrogen fertilisation in the form of low-biuret urea foliar sprays increased flowering of moderately stressed trees in one trial. Varying climatic conditions complicated efforts to induce enough WD stress consistently and subsequent floral stimulation. Furthermore, a high rate of floral abscission resulted in only one trial achieving a significant increase in *forzatura* flowering for the late summer/autumn period overall. There was no significant effect of the WD stress treatments on the internal quality of fruit from the winter crop. No significant changes in expression of the key citrus flowering genes *FLOWERING LOCUS T* (*FT*), *APETALA 1* and *AGOMOUS* was observed, although a non-significant (*P* = 0.0861) increase in the expression of *FT* was observed in buds of extensively stressed trees compared with irrigated trees.

ADDITIONAL INDEX WORDS: *APETALA1*, floral induction, *FLOWERING LOCUS T*, *forzatura*, stem-water potential, *Verdelli*, water-deficit stress

4.1. Introduction

Lemon (*Citrus limon*) trees grown in subtropical climates flower profusely in spring (Saunt, 2000). An additional flowering reaction can occur in autumn, but is usually smaller compared to the spring flowering and the crop is usually considered commercially insignificant (Calabrese and Di Marco, 1981). However, the intensity of this flowering reaction can be manipulated using various cultural manipulation techniques. The autumn bloom leads to the development of *Verdelli* lemons (Raveh, 2008), which are harvested at a green colour during the following summer. These fruit have a rough, thick peel, are roundish in shape, and usually have a few seeds that are almost completely aborted (Barbera et al., 1985). The market value of the *Verdelli* crop can be up to ten times greater than the winter crop due to a lack of sufficient supply of lemons in a period of high consumer demand (Raveh, 2008).

The *forzatura* technique has been used for many years in the Sicilian lemon industry (Barbera and Carimi, 1988). In this practice growers attempt to maximise the late summer or autumn flowering reaction of lemon trees to increase the volume of the *Verdelli* crop. This technique is also applied by lemon growers in Israel, Spain and California, and by lime (*C. latifolia* Tan.) growers in Florida and Egypt (Davies and Albrigo, 1994; Goodall and Silveira, 1981; Spiegel-Roy and Goldshmidt, 1996;). In the *forzatura* technique, producers impose controlled water-deficit (WD) stress to lemon trees by withholding irrigation for a period of between 4 to 8 weeks in summer, until trees exhibit visual wilting symptoms that do not disappear during the night (Barbera et al., 1985). This stress stimulates reproductive development and flowering is usually visual 3 to 4 weeks upon re-irrigation (Crane, 2004). Sicilian lemon growers have perfected the *forzatura* technique by determining optimum water potentials for stressed trees, as well as applying additional nitrogen (N) fertilisation upon re-irrigation to supplement flowering (Barbera et al., 1985).

Nitrogen-based fertilisers have been proven to enhance flowering of citrus trees exposed to floral induction treatments such as low temperature or WD stress (Barbera et al., 1985; Hake, 1995; Lovatt et al., 1988; 1992). Lovatt et al. (1988) proposed that N fertiliser increases flowering of WD stressed trees by enhancing the stress-related accumulation of ammonia-N, which, in turn, causes the elevated biosynthesis of polyamines such as arginine. These polyamines are important for meristematic activity during flower bud differentiation (Lovatt et al., 1988).

Gibberellin biosynthesis inhibitors, such as triazoles, act by blocking the conversion of ent-kaurene to ent-kaurenoic acid in the GA-biosynthesis pathway in shallots (Saos et al., 2002). The reduced GA-biosynthesis restricts vegetative growth, leading to a greater flowering reaction (El-Otmani et al., 2000). Triazoles, such as uniconazole and paclobutrazol (PBZ), can inhibit endogenous GA-biosynthesis and increase flowering of citrus trees (El-Otmani et al., 2000; Monselise and Halevy, 1964; Monselise et al., 1966). Therefore, they may also have the potential for enhancing an autumn flowering reaction in combination with controlled WD stress during summer in lemons. Commercial use of triazoles, however, has been limited due to reports of reduction in fruit size and yield as well as a strong environmental influence (El-Otmani et al., 2000).

In the model plant *Arabidopsis thaliana*, the gene *FLOWERING LOCUS T* (*FT*) is known to play a central role in triggering the flowering cascade, specifically by integrating different floral pathways and stimulating the expression of floral meristem identity genes such as *APETALA1* (Abe et al., 2005; Horvath, 2009; Parcy, 2005; Taiz et al., 2018). The expression of these floral meristem identity genes leads to the up-regulation of floral organ identity genes such as *AGAMOUS* (Taiz et al., 2018). It has been proven that the homologs of these genes function similarly in citrus and other woody species (Endo et al., 2005; Nishikawa et al., 2009; Pillitteri et al., 2004; Tan and Swain, 2007; Tang and Lovatt, 2019).

Research evaluating the effect of WD stress on the expression on citrus flowering genes is limited. Chica and Albrigo (2013) reported that WD stress resulted in the up-regulation of *FT* together with an increased flowering reaction, and that the expression of *AP1* only increased after the trees were re-irrigated and floral development commenced. In contrast, Tang (2017) reported no effects of WD stress on the expression of *FT* but a significant increase in *AP1* expression in stressed trees.

In this chapter we evaluate the hypothesis that *FT* and *AP1* is up-regulated when trees receive WD stress, similar to the way in which these genes respond to low temperature floral-stimulation. The main aim of this study was to evaluate the potential of adapting the *forzatura* technique to South African conditions and the effect of controlled WD stress on out-of-season production of lemons Additionally, an attempt was made to determine the sequence of flowering in this period and evaluate the effect of WD stress treatment on the expression of

genes known to be integral to the floral cascade. Furthermore, the effect of uniconazole or nitrogen foliar applications in combination with WD stress treatment on external and internal fruit quality was evaluated.

4.2. Materials and methods

4.2.1. Plant material and experimental sites

'Lisbon' lemon, De Doorns

The experiment was conducted during the 2017/2018 season in De Doorns (33°51'S 19°51'E), South Africa, on non-bearing four-year-old 'Lisbon' lemon trees budded onto 'Carrizzo' citrange (*C. sinensis* × *Poncirus trifoliate* L. Raf.) rootstock and planted in a shale soil. The orchard was planted in a North-to-South row direction at a spacing of 5 m between rows and 2 m between trees and irrigated using a single-line drip irrigation system. Each dripper supplied water at 2.3 L water per hour, and each tree received approximately 2000 L water per annum. During the following season (2018/2019), the same orchard was used for an additional experiment. However, an additional dripper line was installed in each planting row so that trees received approximately 4000 L per annum.

'Eureka seedless' lemon, Stellenbosch

The experiment was conducted during the 2018/2019 season in Stellenbosch (33°95'S 18°88'E), South Africa, on non-bearing two-year-old 'Eureka seedless' lemon trees budded onto 'Rough lemon' [*C. Jambiri* (Lush.)] rootstock and planted in a clay/loam soil. The orchard was orientated in a North-to-South row direction at a spacing of 5 m between rows and 3 m between trees and irrigated using a micro-irrigation system. Each micro-sprinkler irrigated at a rate of 40 L water per hour, which resulted in trees receiving approximately 4200 L water per annum.

'Bearss' lime, Citrusdal

The experiment was conducted during the 2018/2019 season in Citrusdal (32°44'S 19°03'E), South Africa, on eight-year-old 'Bearss' lime (*C. latifolia* Tan) trees budded onto 'Rough lemon' rootstock and planted in sandy soil. The orchard orientation was in an East-to-West row-direction at a spacing of 6 m between rows and 3 m between trees and are irrigated using

a micro-irrigation system. Each micro-sprinkler irrigated at a rate of 120 L water per hour, which resulted in each tree receiving approximately 8000 L water per annum.

4.2.2. Treatments and experimental design

The experimental design for all trials consisted of a randomised complete block design with seven replicates (trees) per treatment in experiments in De Doorns and Stellenbosch, and six tree replicates per treatment in the experiment in Citrusdal, all in the Western Cape (Mediterranean climate). All replicate trees were selected for uniformity in trunk circumference above the bud union, canopy density, and tree size before treatment application. Buffer trees were located between treatment replicates within the treatment rows. Standard commercial practices aimed at obtaining commercial export quality fruit were followed in all the experimental orchards.

A water-deficit (WD) stress period was imposed as a treatment on designated trees in each block by withholding irrigation either completely or by irrigating at half the recommended commercial rate during midsummer. Trees that received no irrigation (0x) during the WD stress period are called *extensively stressed* and trees that received half of the recommended commercial rate (0.5x) are called *moderately stressed* in further discussion of the results. Control trees received the full commercial rate of water throughout the experiment similar to the rest of the orchard. The extensive stress was imposed by completely blocking drippers or micro-sprinklers that supplied water to the specific tree, while moderate stress was imposed by blocking half of the drippers that supplied water to the specific tree or by replacing original micro-sprinkler heads with ones that supplied half the water volume. After the WD stress period, both extensive- and moderately stressed trees were re-irrigated at the same rate as the control.

'Lisbon' Lemon, De Doorns

During the 2017/2018 season, irrigation was withheld for three weeks for the trees receiving an extensive WD stress treatment from the end of December until early January. Trees were then re-irrigated upon severe leaf wilting. Moderate WD stress treatments were applied for six weeks. Three weeks into the moderate WD stress period trees received a uniconazole soil drench treatment (Sunny® 50 SC, Valent BioSciences®; containing 50 $g \cdot L^{-1}$ active ingredient uniconazole-P) or foliar nitrogen (N) [46% low-biuret urea, Nitrophoska (Pty) Ltd,

Stellenbosch, South Africa] spray treatment, respectively. The uniconazole treatment was intended to decrease the production of floral-inhibiting gibberellic compounds and the low-biuret (LB) urea treatment, to increase the ammonia-nitrogen content in buds. The LB urea foliar spray treatment was applied with a motorised backpack sprayer (STIHL, Pietermaritzburg, South Africa) at a dosage rate of 1 kg LB urea per 100 L water and approximately 4 L spray mixture per tree. The uniconazole treatment was applied with a 1 L water volume around the tree trunk with a watering can after scraping away all the leaf debris. A summary of the treatments is shown in Table 4.1.

During the 2018/2019 season, drippers were blocked for a considerably longer period compared to the previous season (18 vs. 6 weeks) for both WD stress intensities, as extensively stressed trees never displayed any visual signs of WD stress, nor did stem water potential measurements indicate any considerable WD stress levels. Unusually high levels of precipitation were measured in this area during the WD stress period. Five treatment combinations with moderate stress were applied, depending on the rate of LB urea treatment and the application of a double-volume irrigation (2x) treatment after the WD stress period (Table 4.2). The LB urea treatment was applied either once or three times, as to ensure a high ammonia-nitrogen content in buds during the WD stress period. The double-volume irrigation treatment was applied to ensure that trees promptly and wholly exited the stressed state. Double-volume irrigation treatments were applied by replacing original micro-sprinkler heads with ones delivering double the volume of water.

'Eureka seedless' lemon, Stellenbosch

During the 2018/2019 season, irrigation was modified to induce WD stress for a longer period (9 weeks for both extensive and moderate WD stress treatments, compared to 3 and 6 weeks for extensive and moderate WD stress treatments, respectively, in the 2017/2018 season). Treatments were applied similar to the 2018/2019 De Doorns trial (Table 4.2) except that no double-volume (2x) irrigation treatments were applied after the WD stress period.

'Bearss' Lime, Citrusdal

During the 2018/2019 season, the extensively stressed trees were not irrigated for 6 weeks, leading to visual wilting symptoms (Fig. 4.1), while the moderate stress was imposed for 9 weeks. In a separate treatment, 300 g limestone ammonium nitrate (LAN, 28% nitrogen,

Kynoch, Fourways, South Africa) was applied to moderately stressed trees after 4 and 9 weeks of WD stress. A summary of treatments is shown in Table 4.3.

4.2.3. Data collection

4.2.3.1. Stem water potential

Water stress was quantified by measuring midday stem water potential (SWP) with a pressure bomb (Model 600; PMS Instrument Co., Albany, OR) at three-week intervals, from the start of the WD stress period up to three weeks thereafter. In each replicate, three mature, healthy leaves were covered with aluminium foil an hour before measurement to allow for equilibration of the plant water status in experimental leaves with that of the whole-tree plant water status.

4.2.3.2. Gene expression

Sample collection

In the Stellenbosch trial, the first five apical buds from the terminal position of five non-bearing shoots per tree were collected one day before treatment (extensive WD stress) and 9 weeks thereafter. The same sampling times applied for the untreated control. Collected buds were promptly placed in a plastic bag in an ice-filled cooler box for immediate transport to the laboratory. Bud samples were finely ground in liquid nitrogen using mortar and pestle, and promptly stored at -80 °C until further analysis.

RNA extraction, cDNA synthesis, qPCRs and gene expression data analysis were performed the same as in Chapter 3 (p. 37). The sequences of forward and reverse primers of target and reference genes are shown in Table 4.4.

4.2.3.3. Bud development

Ten non-fruiting and purely vegetative shoots 10-15 cm in length were selected and tagged on each replicate tree on the day of the first foliar spray treatment. The numbers of inflorescences and vegetative shoots that sprouted from each tagged shoot were recorded at three-week intervals after WD treatment until late autumn. The mean number of flowers, new vegetative shoots, and fruit per branch per treatment were determined for statistical analysis.

4.2.3.4. Winter fruit quality

During the harvest of the commercial winter crop, ten fruit were collected from random positions within the tree canopy of each treatment replicate. Five of the 10 fruit were immediately analysed for treatment effects on commercial internal and external quality attributes, while the rest were stored for 30 d at 3 ºC. After the cold storage period, fruit were stored at room temperature for five days to allow for the development of any chilling injury (CI) symptoms, after which quality parameters were analysed.

For external quality parameters, fruit rind colour was determined using the CRI colour chart for lemons [no. 37, Citrus Research International (CRI), 2004]. The colour grades ranged from completely yellow (colour grade 1) to completely green (colour grade 8). Fruit diameter was measured using an electronic calliper (CD-6” C, Mitutoyo Corp, Tokyo, Japan). The chilling injury was quantified by evaluating the surface area displaying CI symptoms (sunken lesions or discolorations) and grading the extent of CI on a severity scale of 0 to 3, with 0 having no CI and 3 displaying extensive CI symptoms. A CI index was then calculated for each replicate tree by adding the products of the number of fruit in each CI severity group (0-3) by the severity group number in which it was classed.

Fruit were longitudinally cut in half for internal quality evaluations and juiced (Sunkist®, Chicago, USA), before being strained through a muslin cloth. The juice content was determined by dividing the weight of the juice by the total fruit weight and calculating the juice content as a percentage value. An electronic refractometer (PR-32 Palette, Atago Co, Tokyo, Japan) was used to determine total soluble sugars (TSS) from the extracted juice. Titratable acidity (TA) expressed as citric acid content was determined by titrating 20 mL of the extracted juice against 0.1 N sodium hydroxide using phenolphthalein as an indicator. The TSS:TA ratio was calculated by dividing the TSS values by the TA values.

4.2.4. Statistical analysis

Statistical analyses of variance (ANOVA) were performed using STATISTICA [Dell Inc. 2015, Dell Statistica (Data analysis software system) version 13. software.dell.com]. Repeated measures analyses of variance (RMANOVA) were used to test for treatment effects on the number of inflorescences and vegetative shoots per tree over time. Mean separations were carried out using the least significance difference test (Fisher’s LSD). A *P* value significance threshold of 0.05 was selected in all instances.

4.3. Results

4.3.1. Stem water potential

'Lisbon' lemon, De Doorns

During the 2017/2018 season, midday stem water potentials (SWP's) measurements for control and 0.5x trees showed high levels of moisture stress by the end of the WD stress period (Week 6) (Table 4.5). At this time, 0.5x trees had significantly ($P < 0.0001$) lower SWP's compared with the control trees, with a mean SWP difference of 0.88 MPa (Table 4.5). Three weeks later, both sets of trees exhibited considerably higher SWP's (> 1.6 MPa), with that of the control trees being significantly higher than 0.5x trees (Table 4.5).

During the 2018/2019 season, there were no differences in SWP immediately preceding the WD stress period (Table 4.5). In the following weeks, SWP's were relatively high compared to the 2017/2018 season, with SWP never dropping below -1.65 MPa for all treatments (Table 4.5). The 0x treatment consistently caused trees to exhibit statistically lower SWP's compared to control trees (Table 4.5). The 0.5x treatment, however, had no significant effect on SWP in comparison with the control (Table 4.5).

'Eureka seedless' lemon, Stellenbosch

High SWP's above -0.7 MPa were recorded immediately preceding the WD stress period, with no significant differences between treatments (Table 4.6). In week three, the average SWP of 0x trees was significantly lower than 0.5x and control trees, while 0.5x trees did not exhibit lower SWP's than control trees (Table 4.6). By week 6, the three treatments' SWP's differed significantly ($P < 0.0001$) from one another, with 0x trees displaying the lowest SWP of almost -2 MPa, and control trees having the highest SWP (Table 4.6). In week 9, 0x trees had a significantly lower SWP than control trees, although not significantly lower than 0.5x trees (Table 4.6). By week 12 (after re-irrigation), SWP's for the three treatments were between -0.85 and -0.93 MPa and did not differ significantly from one another (Table 4.6).

'Bearss' lime, Citrusdal

Midday SWP's did not differ significantly between treatments immediately preceding the start of the WD stress period (Table 4.7). By week 3, the average SWP of 0x trees (-1.76 MPa) was significantly lower than for control trees (Table 4.7). The average SWP for 0.5x trees did not

differ significantly from control trees or 0x trees (Table 4.7). Extensive stressed trees exhibited highly negative SWP's (< -3 MPa) in week 6 of the WD stress treatment, significantly lower than 0.5x and control trees, which did not differ statistically from each other (Table 4.7). In week 9, the average SWP for 0.5x trees (-1.8 MPa) was significantly lower than control trees and 0x trees, which did not differ significantly from each other (Table 4.7). By week 12, the average SWP of the three treatments did not differ significantly from one another (Table 4.7).

4.3.2. Gene expression

There was no significant difference between the relative expression of *FT* and *AP1* between buds of 0x and control trees immediately preceding the onset of the WD stress period, or after 9 weeks of WD stress (Fig. 4.2). However, the average *FT* expression of buds on 0x trees was notably higher ($P = 0.0861$) than control trees in week 9 of the WD stress period (Fig. 4.2).

4.3.3. Bud development

'Lisbon' lemon, De Doorns

In the period after WD treatments, very little vegetative growth was observed in all trees in the 2017/2018 season (Fig. 4.3). The number of vegetative shoots that sprouted in the WD stress treatments was significantly lower ($P < 0.0001$) compared with the control trees and sprouted a minimum of 42% less new vegetative shoots compared to control trees (Table 4.8).

The 0x and 0.5x + LB urea treatment resulted in significantly more flowers per shoot compared to the control in week 6 of the WD stress period (Fig 4.3). Floral abscission was especially prevalent in 0x trees after week 6 (Fig. 4.4). Between weeks 15 and 18, all trees exhibited a flowering response, with the 0.5x treatment flowering at a significantly higher rate compared with the control and 0x treatments (Fig 4.3). For the period mid-summer to late autumn, 0x and 0.5x + uniconazole or LB urea treatments flowered significantly more compared to control trees (Table 4.8). However, the 0.5x treatment without uniconazole or LB urea did not affect flowering compared to the control (Table 4.8). Moderate WD stress + LB urea was the only treatment to cause a significant increase in flowering compared to both 0.5x and control trees (Table 4.8). However, the treatments did not affect the eventual fruit set compared to the control (Table 4.8).

During midsummer to late autumn of the 2018/2019 season, bud growth of all trees was extremely limited and similar (Table 4.9).

'Eureka seedless' lemon, Stellenbosch

No clear vegetative growth pattern was observed in trees during the midsummer to late autumn periods (Fig. 4.5). Extensive stressed and 0.5x trees consistently sprouted the lowest number of vegetative shoots (Fig. 4.5), with the 0.5x trees significantly sprouting the least vegetative shoots for the period midsummer to late autumn, although not significantly less than 0x trees (Table 4.10). The 0.5x + 3 LB urea + 2x treatment caused sprouting of the highest number of vegetative shoots per branch over this period, although not significantly higher than that of the control (Table 4.10).

Two general flowering reactions were observed for all trees; in the 6th week after the start of the WD stress period and from week 12 to 15 (Fig. 4.5). The largest differences in flowering between treatments were observed in week 12, where 0.5x + 1LB urea trees and 0.5x + 2x trees exhibited increased flowering compared with control trees (Fig. 4.5). This was the only period where treated trees displayed a higher rate of flowering than control trees. For the period midsummer to autumn, the only treatment that caused a significantly higher rate of flowering compared to the control was the 0.5x + 2x treatment (Table 4.10). Extensive stressed trees flowered at a significantly decreased rate compared to control trees (Table 4.10). However, the treatments did not affect the eventual fruit set significantly compared with the control (Table 4.10).

'Bearss' lime, Citrusdal

Control trees consistently sprouted new vegetative shoots, whereas stressed trees only sprouted new shoots from week 12 after the start of the WD stress period (Fig. 4.6). However, only in week 15 did control trees have a significantly higher number of shoots per branch compared with stressed trees (Fig. 4.6). Overall, control trees exhibited a higher rate of vegetative growth than stressed trees, sprouting at least twice the number of shoots in the period midsummer to late autumn (Table 4.11).

Extensive stressed trees exhibited a strong flowering reaction in week 9, where they flowered at a significantly higher rate compared to the other treatments, before rapidly losing flowers by week 12 (Fig. 4.6). In week 15, a marked flowering reaction was observed for all trees except 0x trees. The 0.5x + LAN treatment had a significantly higher number of flowers per branch compared to any other treatment in week 15, although the number of flowers quickly decreased

by week 18 (Fig. 4.6). Overall, there were no significant differences in the number of flowers per branch between treatments for the period midsummer to late autumn (P = 0.0744) (Table 4.11). However, the 0.5x with LAN treatment increased flowering by an average of 88% compared to the control. The treatments did not affect the eventual fruit set significantly compared with the control (Table 4.11).

4.3.4. Winter fruit quality

'Eureka Seedless' lemon, Stellenbosch

There were no significant differences in external quality parameters between treatments in fruit at harvest or after cold storage (Table 4.12). There was, however, a significantly higher juice percentage measured in 0x trees after cold storage (Table 4.12). No significant difference in chilling injury prevalence between treatments were observed (data not shown)..

'Bearss' lime, Citrusdal

There were no significant differences in internal fruit quality parameters between treatments at harvest or after cold storage except for fruit size and fruit weight, with control trees displaying higher values in both instances (Table 4.13). No significant difference in chilling injury prevalence between treatments were observed (data not shown).

4.4. Discussion

In treatments where controlled WD stress managed to reduce SWP to values lower than -2.5 MPa, such as in lemon trees in De Doorns in 2017/2018 and lime trees in Citrusdal, a considerable flowering response was obtained three to six weeks after re-irrigation. This concurs with previous studies and observations of the practice by Sicilian lemon growers (Burke, 1951; Crane, 2004; Goodall and Silveira, 1981). However, although flowering was to some extent achieved by the *forzatura* technique, the total number of flowers during the complete midsummer/autumn period in all trials was not consistently higher compared with the control. In addition, whenever a significant flowering reaction was achieved after sufficient stress, flowers abscised rapidly and fruit set was poor, similar to reports by Goodall and Silveira (1981) in studies with 'Persian' lime. As expected, there was no difference in the number of fruit that set and the eventual size of the *Verdelli* crop.

The *forzatura* technique originates from the lemon-producing areas of Sicily, where soils are mostly shallow volcanic ash with a low water-holding capacity (Burke, 1951; Stander, 2018). Sicilian growers experience typical Mediterranean-type climate with cold, wet winters and warm, dry summers (Hake, 1995). The dry and warm climatic conditions during summer in combination with soils that easily dry out are ideal for producers to successfully apply the *forzatura* technique. However, the technique, as practiced in Sicily does not invariably provide a substantial summer crop. Growers struggle to achieve sufficient autumn flowering in uncharacteristically cold and/or rainy summers which result in a restricted or non-existent summer crop (Giancarlo Roccuzo, personal communication). Furthermore, the most effective treatments to induce summer flowering were experiments conducted on potted trees and in greenhouse conditions where soil water levels can be precisely regulated (Chica and Albrigo, 2013; Southwick and Davenport, 1986; Tang, 2017).

In the current study, a consistent *forzatura* flowering reaction was not achieved. A large obstruction to the successful application of the *forzatura* technique is varying soil- and climatic conditions such as unanticipated summer rains or soils with a high water-retention capacity (Burke, 1951; Crane, 2004; Goodall and Silveira, 1981). These factors negate the effects of controlled WD stress on floral development and subsequent success of the out-of-season flowering response. In this study, climatic conditions varied greatly between seasons: the 2017/2018 season was characterised by a hot, dry summer and the following summer by frequent precipitation. Additionally, the summer was preceded by a wet winter. Subsequently, in the De Doorns trial where the orchard is located in an area that receives constant run-off from mountainous streams, the extensive stress treatment failed to decrease SWP of trees to below -1.7 MPa after 12 weeks of severe WD stress. Therefore, no significant flowering reaction was observed in this trial site for the 2018/2019 season. Goodall and Silveira (1981) reported a similar lack of a wilt response under Californian conditions due to soils being too retentive.

The second reason for failure to consistently reproduce the *forzatura* technique in the current study was the high rate of flower abscission, observed after low soil water levels provided sufficient WD stress to induce a significant flowering reaction. The observed abscission could be ascribed to several possible factors weakening trees' ability to set fruit. These may include decreased photosynthetic assimilates (Goldschmidt, 1999), insect damage (Childers, 1992) and wind (Davies and Albrigo, 1994). Decreased levels of carbohydrates are related to limited fruit

set (Goldschmidt, 1999) and water-deficits lead to a down-regulation of photosynthesis (Chaves et al., 2002). Therefore, a reduction in photosynthetic assimilates coupled with an increase in flowers that develop in a period during which roots and new shoots develop rapidly, may lead to increased inter-sink competition and a subsequent elevated abscission rate.

Not only can wind and insects aggravate tree stress by affecting vapour pressure deficits and photosynthesis, but could directly cause floral damage (Childers, 1992). In the current study, floral insect pests such as citrus thrips (*Scirtothrips aurantii*) were observed in the orchards (personal observations). These pests were not chemically controlled due to export residue limitations on chemical control options of the winter crop. A study by Childers (1992) showed that increased suppression of flower thrips [*Frankliniella bispinosa* (Morgan)] during bloom in Florida was positively correlated with 'Navel' orange fruit set. Therefore, high wind speeds and increased insect damage, characteristic of Western Cape summers, could be a potential drawback to the implementation of the *forzatura* technique by reducing fruit set of the mid-summer flowering reaction, especially if the summer insect pest complex is not managed to protect the out-of-season flowering.

Mid-summer urea sprays to WD stressed trees aimed at increasing late summer/autumn flowering did not provide conclusive results. However, in one of the experiments the application of urea to moderately stressed trees increased flowering compared with trees receiving only a moderate stress treatment, as well as the control. These results are similar to studies showing that nitrogen-based fertilisers can supplement flowering of trees exposed to floral stimulating treatments (Barbera et al., 1985; Hake, 1995; Lovatt et al., 1988; 1992). Nonetheless, this has not been proved conclusively in the current study, as similar increases in flowering were not observed across all trial sites. This can possibly be ascribed to insufficient WD stress intensity and/or high temperatures during application leading to decreased uptake of foliar applications. The use of uniconazole to increase late summer/autumn flowering of WD stressed trees increased flowering of moderately stressed trees compared to trees receiving only a moderate stress treatment. It may, therefore, have the potential for commercial use, although additional trials in different seasons and production areas are necessary.

Lemon trees are extremely vigorous and producers are forced to frequently prune trees to increase light penetration and encourage reproductive growth (Davies and Albrigo, 1994). In the current study, the withholding of irrigation during mid-summer, either completely or at half

the normal rate, caused a reduction in vegetative growth in the autumn period. This can be beneficial to producers aiming to decrease vigour in lemon orchards and decrease water usage in the process; especially in drought-prone areas. Also, both WD stress intensities did not affect the internal quality parameters of winter fruit. Similarly, Barbera and Carimi (1988) did not find any adverse effects of mid-summer water-deficit stress on internal quality of lemon fruit.

The alternation of root- and shoot growth in citrus trees is well-known, and the involvement of endogenous hormones in this oscillation has long been hypothesised (Bevington and Castle, 1985; Spiegel-Roy and Goldschmidt; 1996). Water stress restricts root growth of citrus trees (Bevington and Castle, 1985). Therefore, restriction of root growth due to soil-water deficits may lead to a decreased flow of the endogenous hormone cytokinin (CK) to the upper parts of the tree. Subsequently, the initiation of shoot growth may be reduced due to lower concentrations of CK in buds. Additionally, the production of gibberellins from actively growing rootlets (Carr et al., 1964; Goldschmidt et al., 1997) may decrease during drought conditions, leading to a diminished inhibition of reproductive bud development (Lord and Eckard, 1987).

Severe water-deficit stress did not affect the expression of *FT* or *AP1*. This is in contrast to Chica and Albrigo (2013) and Tang (2017), who found increased mRNA levels of *FT* and *AP1*, respectively. The relative expression of *AG* was too low to be quantified in the current study. It should be noted that in the severe water-deficit stress experiment where gene expression was evaluated, there was no significant effect of WD stress treatments on flowering, which explains the lack of difference in the expression of flowering genes between treatments. There was, nonetheless, a non-significant (P = 0.0861) increase in the expression of *FT* in buds of extensively stressed trees compared with the control, whereas mRNA levels of *AP1* were similar between treatments. This may lead to the possibility that relative expression of *FT*, although increased, was not sufficient to incur considerable changes in protein production to stimulate the rest of the flowering cascade and phenotypic changes.

The cultivars used in this study, i.e., ‘Lisbon’ and ‘Eureka Seedless’ differ from cultivars that are primarily used in Sicilian citriculture, especially the ‘Feminello’ cultivar which is characteristically ever-blooming and –bearing and thus very responsive to forced treatments such as the *forzatura* technique (Sinclair, 1984). This could have contributed to the failure in this study to consistently reproduce the technique. The use of the ‘Feminello’ cultivar in this

study, however, would not be justified as it is of low economic importance in the South African industry.

In conclusion, an attempt was made to reproduce the *forzatura* technique under South African conditions. There were varying degrees of success, but consistent stimulation of flowering could not be achieved, primarily due to climatic variability leading to insufficient WD stress intensities. In cases where floral stimulation was indeed successful, a high rate of abscission was observed, likely due to photosynthetic down-regulation by the WD stress and wind- and insect damage to flowers. Therefore, additional studies over a longer period are necessary to determine if this technique can be employed in South Africa. Furthermore, the growth and yield of the *Verdelli* summer fruit need to be evaluated to eventually assess the financial benefits of a summer crop versus a winter crop.

4.5. Literature cited

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Table 4.1. Summary of water-deficit (WD) stress treatments applied to 'Lisbon' lemons at De Doorns, Western Cape, South Africa, during the 2017/2018 season.

Treatment	Water-deficit (WD) stress period	LB urea[z] application	Uniconazole[y] application
Control	-	-	-
1	0x[x] for 3 weeks	-	-
2	0.5x for 6 weeks	-	-
3	0.5x for 6 weeks	Week 3[w]	-
4	0.5x for 6 weeks	-	Week 3

[z] Low-biuret urea (46% nitrogen).

[y] Sunny® 50 SC (Valent BioSciences®, 50 $g \cdot L^{-1}$ active ingredient Uniconazole-P).

[x] Fraction of control irrigation volume.

[w] Weeks after the start of WD stress period.

Table 4.2. Summary of water-deficit (WD) stress treatments applied to 'Eureka' lemons at Welgevallen experimental farm, Stellenbosch, Western Cape, South Africa, and to 'Lisbon' lemons at De Doorns, Western Cape, South Africa, during the 2018/2019 season.

Treatment	Water-deficit (WD) stress treatment	LB urea[z] treatment	2x[y] treatment after WD stress period
Control	-	-	-
1	0x	-	-
2	0.5x	-	-
3	0.5x	Week 3[x]	-
4	0.5x	Week 3, 6 and 9	-
5	0.5x	-	For 3 weeks
6	0.5x	Week 3, 6 and 9	For 3 weeks

[z] Low-biuret urea (46% nitrogen).

[y] Fraction of control irrigation volume.

[x] Weeks after the start of WD stress period.

Fig. 4.1. Visual wilt symptoms of ‘Bearss’ lime trees in reaction to extensive water-deficit stress at Citrusdal during the 2018/2019 season.

Table 4.3. Summary of water-deficit (WD) stress treatments applied to 'Bearss' lime at Citrusdal, Western Cape, South Africa, during the 2018/2019 season.

Treatment	Water-deficit (WD) stress period	LAN[z] application
Control	-	-
1	0x[y] for 6 w	-
2	0.5x for 9 w	-
3	0.5x for 9 w	Week 4, 9[x]

[z] Limestone ammonium nitrate (28% nitrogen).

[y] Fraction of control irrigation volume.

[x] Weeks after the start of WD stress period.

Table 4.4. Forward and reverse primer sequences for the citrus target and reference genes used in the quantitative real-time PCR (qPCR) assays.

Gene abbreviation	Annotation	Forward primer (5' to 3') Reverse primer (5' to 3')	Amplicon length (bp)	Primer concentration (µM)	Mean efficiency (%)	Reference
FT	Flowering locus T	CCGCGTTGTTGGTGATGTTCTTGA ATTTCAGCCCTAGGCTGGTTCAGA	132	0.25	83	Tang and Lovatt (2019)
AP1	Apetala 1	ACCGCTCTCAAACACATCAG GCAGCCTCTCTCTCTCC	137	0.25	72.5	Tang and Lovatt (2019)
AG	Agamous	GGGAAGTTGACTTGCACAACAGCA TAGCTCCGGGAATCAAATGGCTGA	142	0.25	NA[z]	Tang and Lovatt (2019)
ACT	Actin	TCACAGCACTTGCTCCAAGCAG TGCTGGAAGGTGCTGAGGGA	130	0.25	83.5	Tang and Lovatt (2019)
GAPC2	Glyceraldehyde-3-phosphate dehydrogenase C2	TCTTGCCTGCTTTGAATGGA TGTGAGGTCAACCACTGCGACAT	80	0.25	68	Mafra et al. (2012)

[z] Expression value below the threshold for detection (quantitation cycle [Cq] in qPCR > 35).

Table 4.5. The effect of mid-summer water-deficit (WD) stress treatments on mean Midday Stem Water Potential (MPa) of 'Lisbon' lemon trees at De Doorns, Western Cape, South Africa,during the 2017/2018 and 2018/2019 seasons.

Treatment	Average Midday Stem Water Potential (MPa)							
	Week 0[z]		Week 3		Week 6		Week 9	
Year	2017/2018	2018/2019	2017/2018	2018/2019	2017/2018	2018/2019	2017/2018	2018/2019
Control	-[y]	-1.26[ns]	-	-1.13a[x]	-2.08a	-1.30a	-1.58a	-1.12a
0.5x[w]	-	-1.14	-	-1.22a	-2.96b	-1.48a	-1.36b	-1.25ab
0x	-	-1.31	-	-1.50b	-	-1.65b	-	-1.37b
P Value		0.06		0.0005	< 0.0001	0.002	0.0142	0.004

[z] Number of weeks after start of WD stress period.

[y] No measurements taken.

[ns] No significant differences.

[x] Means with a different letter within a column differ significantly at the 5% level (least significant difference).

[w] Fraction of control irrigation volume.

Table 4.6. The effect of mid-summer water-deficit (WD) stress treatments on mean Midday Stem Water Potential (MPa) of 'Eureka Seedless' lemon trees at Welgevallen experimental farm, Stellenbosch, Western Cape, South Africaduring the 2018/2019 season.

Treatment	Midday Stem Water Potential (MPa)				
	Week 0[z]	Week 3	Week 6	Week 9	Week 12
Control	-0.67[ns]	-0.77a[y]	-1.22a	-1.20a	-0.93[ns]
0.5x[x]	-0.60	-0.97a	-1.60b	-1.5ab	-0.94
0x	-0.61	-1.30b	-1.98c	-1.76b	-0.85
P Value	0.463	0.002	< 0.0001	0.006	0.124

[z] Number of weeks after start of WD stress treatment period.

[ns] No significant differences.

[y] Means with a different letter within a column differ significantly at the 5% level (least significant difference).

[x] Fraction of control irrigation volume.

Table 4.7. The effect of mid-summer water-deficit (WD) stress treatments on Midday Stem Water Potential (MPa) of 'Bearss' lime trees at Citrusdal, Western Cape, South Africa, during the 2018/2019 season.

Treatment	Midday Stem Water Potential (MPa)				
	Week 0[z]	Week 3	Week 6	Week 9	Week 12
Control	-1.61[ns]	-1.20a[y]	-1.27a	-1.40a	-1.33[ns]
0.5x[x]	-1.72	-1.46ab	-1.70a	-1.80b	-1.29
0x	-1.72	-1.76b	-3.13b	-1.38a	-1.37
P Value	0.696	0.009	< 0.0001	0.046	0.615

[z] Number of weeks after start of WD stress treatment period.

[ns] No significant differences.

[y] Means with a different letter within a column differ significantly at the 5% level (least significant difference).

[x] Fraction of control irrigation volume.

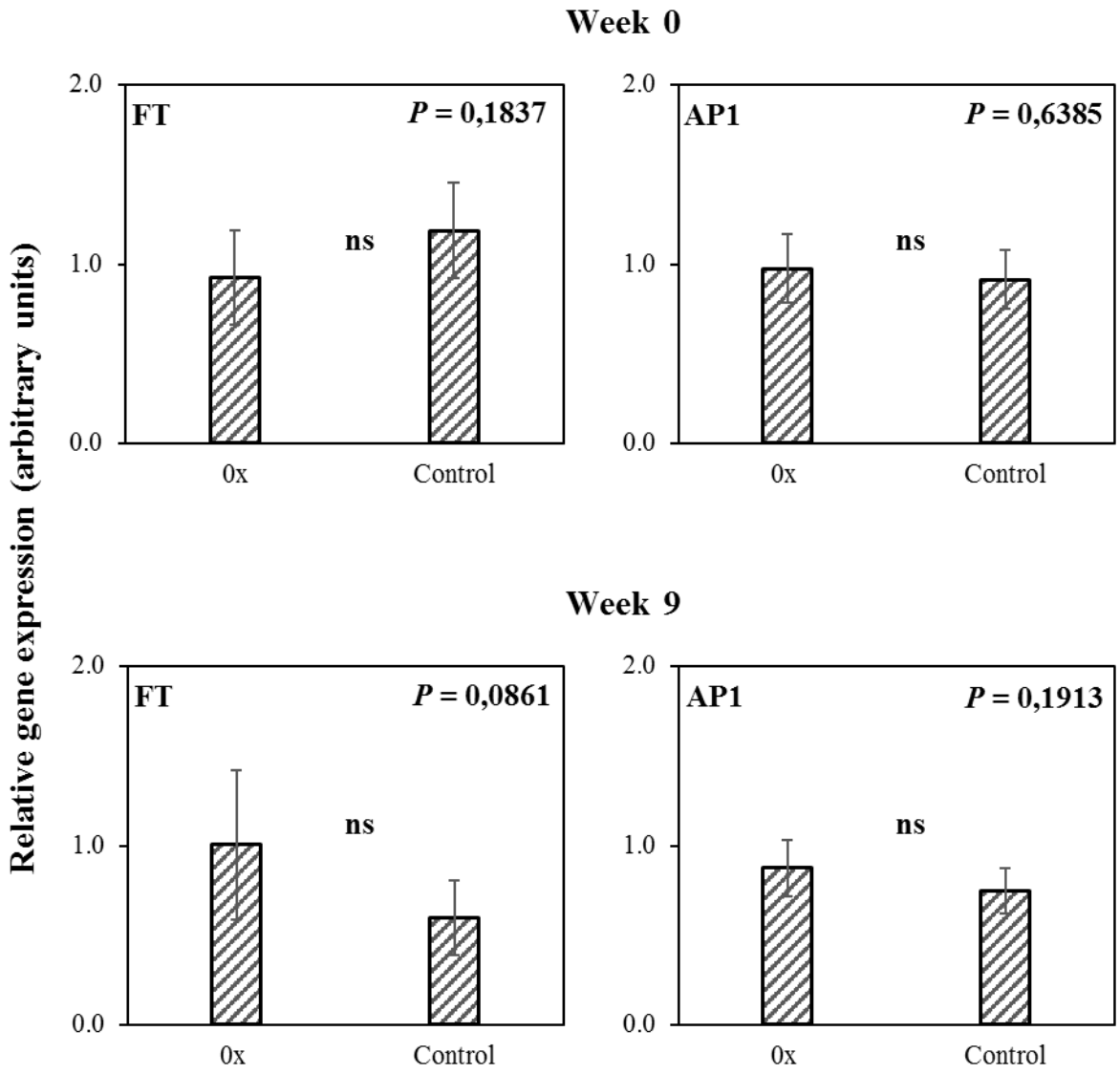

Fig. 4.2. Relative expression of *FT* and *AP1* in buds of 'Eureka Seedless' lemon trees at the Welgevallen Experimental farm, Stellenbosch, Western Cape, South Africa. Week 0 and week 9 indicate sampling on the day of the start of the WD stress treatment and sampling on the last day of the WD stress treatment, respectively. ns = No significant differences between control and treatment means at the 5% level.

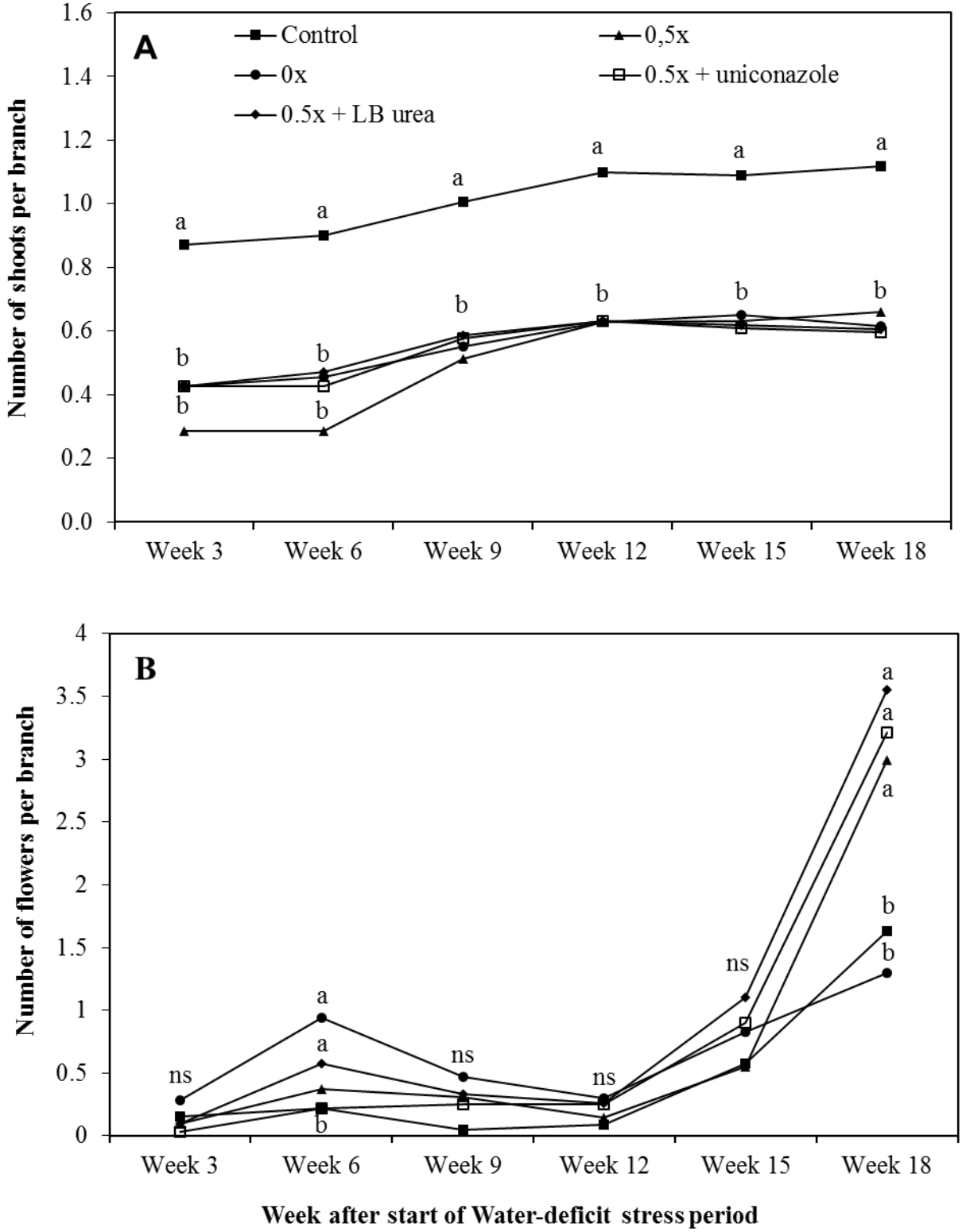

Fig. 4.3. Mean number of vegetative shoots (**A**) and flowers (**B**) per shoot counted from mid-summer to late autumn 2017/2018 on 'Lisbon' lemon trees receiving different stress treatments at De Doorns, Western Cape, South Africa. For the same week, means with a different letter differ significantly at the 5% level (ns = No significant differences).

Table 4.8. The effect of different mid-summer water-deficit (WD) stress treatments on growth of 'Lisbon' lemon trees in mid-summer/autumn of the 2017/2018 season at De Doorns, Western Cape, South Africa.

Treatment	Vegetative shoots per branch	Flowers per branch	Fruit per branch
Control	1.06a[z]	0.45c	0.09ns
0.0x[y] for 3 weeks	0.61b	0.75ab	0.12
0.5x for 6 weeks	0.6b	0.69bc	0.21
0.5x for 6 weeks + uniconazole[x]	0.59b	0.81ab	0.13
0.5x for 6 weeks + LB urea[w]	0.59b	1.0a	0.13
P value	< 0.0001	0.0033	0.2583

[z] Means with a different letter within a column differ significantly at the 5% level (least significant difference).

[ns] No significant differences.

[y] Fraction of control irrigation volume.

[x] Sunny® 50 SC (Valent BioSciences®, 50 $g \cdot L^{-1}$ active ingredient Uniconazole-P)

[w] Low-biuret urea (containing 46% nitrogen).

Fig. 4.4. Floral abscission of water-deficit stressed ‘Lisbon’ lemon trees at De Doorns during the 2017/2018 season.

Table 4.9. The effect of different mid-summer water-deficit (WD) stress treatments on growth of 'Lisbon' lemon trees during mid-summer/autumn of the 2018/2019 season at De Doorns, Western Cape, South Africa.

Treatment	Vegetative shoots per branch	Flowers per branch	Fruit per branch
Control	0.09[ns]	0.03b[z]	0.07b
0.0x[y]	0.02	0.01b	0.02b
0.5x	0.06	0.03b	0.02b
0.5x + LB urea[x]	0.04	0.01b	0.01b
0.5x + 3 × LB urea	0.06	0.15a	0.01b
0.5x + 2x	0.05	0.07b	0.14a
0.5x + 3 × LB urea + 2x	0.04	0.07b	0.06b
P value	< 0.0001	0.0001	< 0.0001

[ns] No significant differences.

[z] Means with a different letter within a column differ significantly at the 5% level (least significant difference).

[y] Fraction of control irrigation volume.

[x] Low-biuret urea (containing 46% nitrogen).

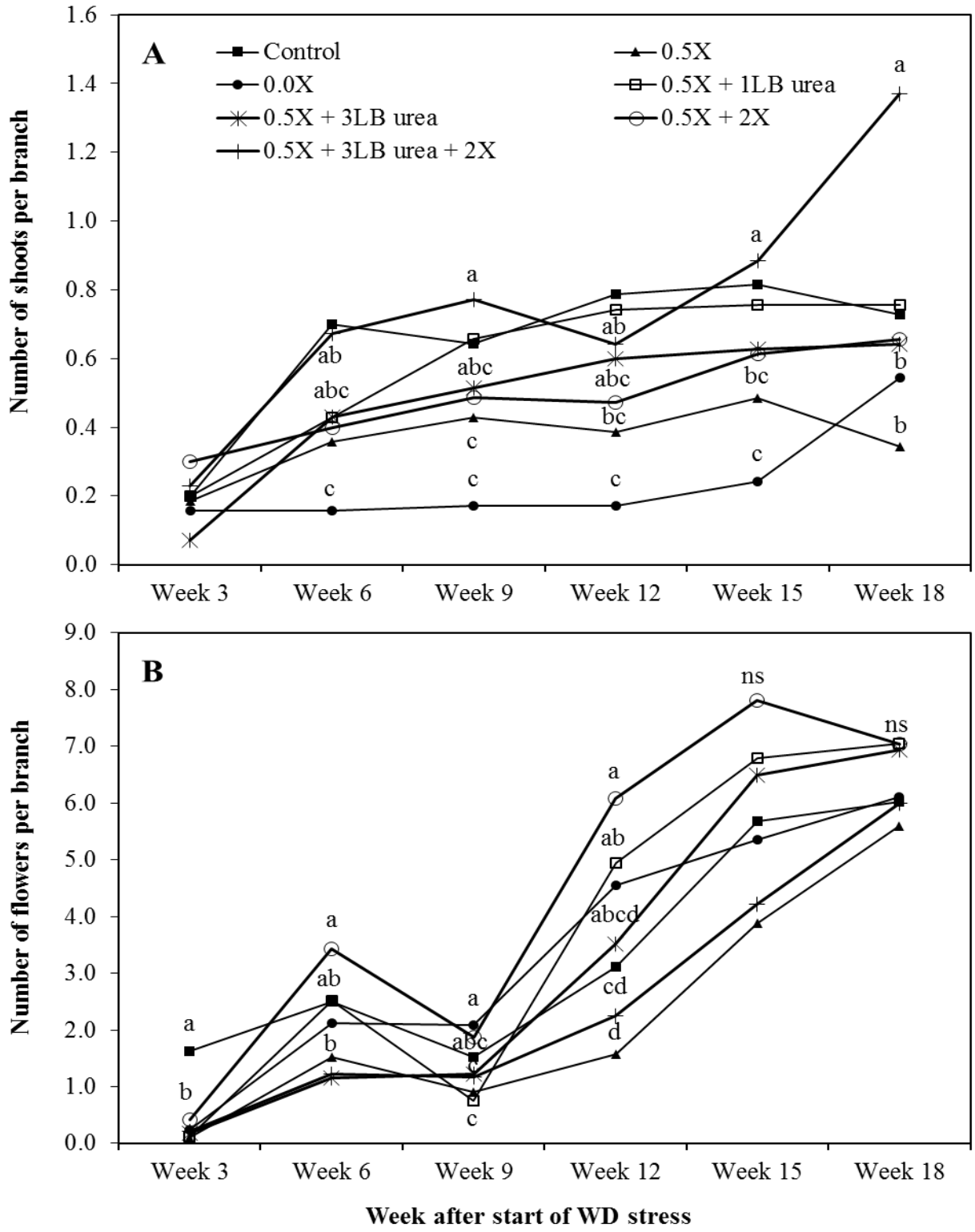

Fig. 4.5. Mean number of vegetative shoots (**A**) and flowers (**B**) per shoot counted from mid-summer to autumn 2018/2019 on 'Eureka seedless' lime trees receiving different stress treatments at Welgevallen experimental farm, Western Cape, South Africa. For the same week, means with a different letter differ significantly at the 5% level (ns = No significant differences).

Table 4.10. The effect of different mid-summer water-deficit (WD) stress treatments on growth of 'Eureka seedless' lemon trees in mid-summer/autumn of the 2019/2020 season at Welgevallen experimental farm, Stellenbosch, Western Cape, South Africa.

Treatment	Vegetative shoots per branch	Flowers per branch	Fruit per branch
Control	0.65ab	3.41bc[z]	0.36abc
0.0x[y]	0.36cd	2.26d	0.22c
0.5x	0.24d	3.46bc	0.5a
0.5x + 1 × LB urea[x]	0.6ab	3.69ab	0.26c
0.5x + 3 × LB urea	0.48bc	3.25bc	0.30bc
0.5x + 2x	0.49bc	4.44a	0.45ab
0.5x + 3 × LB urea + 2x	0.76a	2.56cd	0.23c
P value	< 0.0001	0.0002	0.0019

[z] Means with a different letter within a column differ significantly at the 5% level (least significant difference).

[y] Fraction of control irrigation volume.

[x] Low-biuret urea (containing 46% nitrogen).

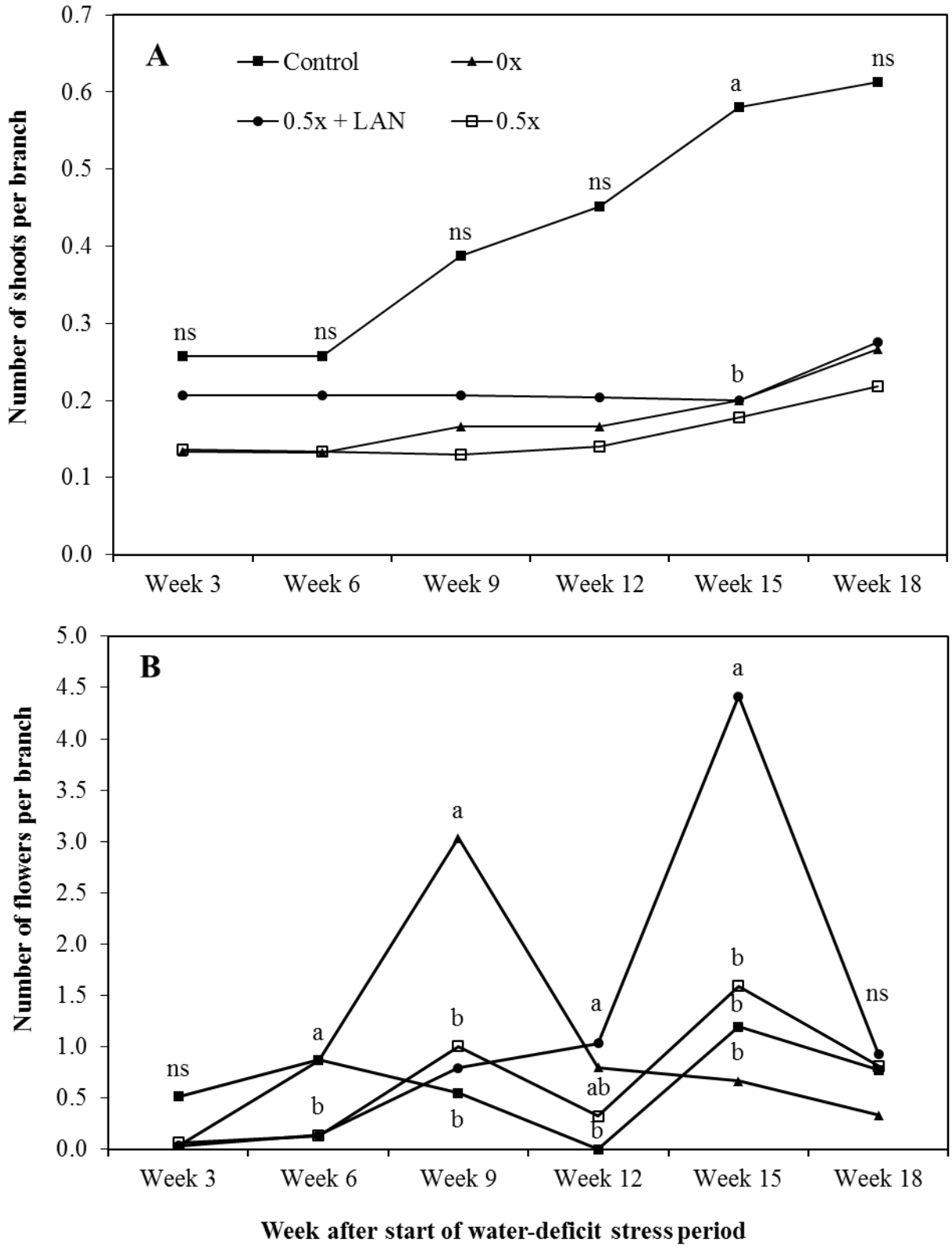

Fig. 4.6. Mean number of vegetative shoots (**A**) and flowers (**B**) per shoot counted from mid-summer to autumn 2018/2019 on 'Bearss' lime trees receiving different stress treatments at Citrusdal, Western Cape, South Africa. For the same week, means with a different letter differ significantly at the 5% level (ns = No significant differences).

Table 4.11. The effect of different mid-summer water-deficit (WD) stress treatments on growth of 'Bearss' lime trees in mid-summer/autumn of the 2019/2020 season at Citrusdal, Western Cape, South Africa.

Treatment	Vegetative shoots per branch	Flowers per branch	Fruit per branch
Control	0.42a[z]	0.65[ns]	0.99[ns]
0.0x[y] for 6 weeks	0.17b	0.96	1.72
0.5x for 9 weeks	0.15b	0.66	1.72
0.5x for 9 weeks + LAN[x]	0.21b	1.22	1.08
P value	0.0349	0.0744	0.0543

[z] Means with a different letter within a column differ significantly at the 5% level (least significant difference).

[ns] No significant differences.

[y] Fraction of control irrigation volume.

[x] Limestone ammonium nitrate (containing 28% nitrogen).

Table 4.12. The effect of extensive stress (0x) treatment on internal and external quality parameters before and after cold storage of 'Eureka Seedless' lemon fruit harvested from Welgevallen experimental farm, Stellenbosch, Western Cape, South Africa.

Treatment	Fruit colour		Fruit size (mm)		Fruit weight (g)		Juice (%)		TSS (ºBrix)		Titratable acidity (%)	
	Day 0[z]	Day 30[y]	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30
Control	4.97[ns]	3.46[ns]	6.99[ns]	6.86[ns]	1013.14[ns]	923.64[ns]	40.80[ns]	41.86b[x]	6.81[ns]	6.80[ns]	4.77[ns]	4.66[ns]
0x	4.77	3.92	6.99	6.73	1015.92	857.75	36.32	46.11a	7.02	7.15	4.83	4.57
P value	0.5752	0.3592	0.9948	0.5477	0.9739	0.1854	0.0885	0.0291	0.5003	0.1823	0.8576	0.6962

[z] Immediately after harvest.

[y] Following 30 d cold storage at 3 ºC.

[ns] No significant differences.

[x] Means with a different letter within a column differ significantly at the 5% level (least significant difference).

Table 4.13. The effect of extensive stress (0x) treatment on internal and external qualities before and after cold storage of 'Bearss' lime fruit harvested from Citrusdal, Western Cape, South Africa.

Treatment	Fruit colour		Fruit size (mm)		Fruit weight (g)		Juice %		TSS (ºBrix)		Titratable acidity (%)	
	Day 0[z]	Day 30[y]	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30
Control	7.23[ns]	6.57[ns]	4.74a[x]	4.58[ns]	339a	302.33[ns]	45.22[ns]	42.78[ns]	9.37[ns]	9.52[ns]	5.82[ns]	5.89[ns]
0x	7.17	6.37	4.41b	4.39	272b	280.92	43.41	42.75	10.13	10.02	6.09	5.87
P value	0.7153	0.3534	0.0323	0.3629	0.0379	0.477	0.3277	0.5723	0.0645	0.115	0.301	0.9599

[z] Immediately after harvest.

[y] Following 30 d cold storage at 3 ºC.

[ns] No significant differences.

[x] Means with a different letter within a column differ significantly at the 5% level (least significant difference).

CHAPTER 5

General discussion and conclusion

An increase in South African lemon plantings (CGA, 2019) could lead to a possible oversupply of lemon fruit during winter and reduced grower returns. By manipulating the natural flowering habit of a lemon tree, the lemon fruit supply peak could be shifted to a period of lesser competition, i.e. summer. This could be achieved by a combination of inhibition of spring flowering (Goldschmidt et al., 1997; Monselise and Halevy, 1964) and stimulation of late summer or autumn flowering (Goodall and Silveira, 1981).

Two gibberellic acid (GA_3) foliar spray applications in early winter (May) inhibited the major lemon and lime flowering response in spring in two seasons, which is in concurrence with results from studies on 'Eureka' lemon (Khelil et al., 2013; Monselise and Halevy, 1964) and 'Washington Navel' sweet orange (Guardiola et al., 1982; Moss, 1970). The lowest concentration of GA_3 foliar sprays, viz. 10 $mg \cdot L^{-1}$, consistently reduced spring flowering compared to untreated, control trees, and to the same extent as 20 and 40 $mg \cdot L^{-1}$ GA_3 treatments. Additionally, GA_3 applications stimulated spring vegetative shoot growth in some cases, similar to what was reported in studies by Guardiola et al. (1982) and Muñoz-Fambuena et al. (2012). Less spring flowers and more spring vegetative shoot growth will increase the potential for summer flowering. Expression of the gene, *FLOWERING LOCUS T* (*FT*), was lower in buds of trees that received foliar GA_3 applications, which concurs with results from studies on 'Salustiana' sweet orange (Muñoz-Fambuena et al., 2012) and 'Orri' mandarin (Goldberg-Moeller et al., 2013). Similarly, mRNA levels of *APETALA1* (*AP1*) was lower in buds after GA_3 treatment compared with untreated control trees, which concurs with results of Tang and Lovatt (2019) in studies on 'Washington navel' sweet orange. As expected, expression of the gene *AGAMOUS (AG)* could not be quantified. Expression of *AG* is reported to be associated with flower development processes much later in the floral cascade (Coen and Meyerowitz, 1991). The inhibitory effect of GA_3 on spring flowering of lemon and lime can be attributed to the inhibition of floral induction in early winter, most likely through down-regulation of *FT* expression, and subsequent effects on the rest of the floral cascade (via *AP1*). We recommend that lemon and lime producers in the Western Cape who wish to reduce intensity of spring flowering should apply two foliar applications of 10 $mg \cdot L^{-1}$ GA_3 in early May, especially in young orchards.

Attempts to adapt the *forzatura* technique under local (Western Cape) production conditions were not consistently successful, most likely due to varying soil characteristics and different climatic conditions across areas and seasons. In cases where soils could not be sufficiently dried out by regulated water deficit (WD) stress, no significant flowering reaction was obtained, which agrees with reports of Goodall and Silveira (1981) on 'Bearss' lime. Nonetheless, where regulated WD stress treatments managed to reduce stem-water potential to values lower than -2.5 MPa, a significant flowering reaction was obtained, three to six weeks after re-irrigation. This concurs with reports on the practice in Sicily where the *forzatura* technique originated (Burke, 1951). However, in WD stress treatments where summer flowering was higher, a high rate of floral abscission occurred, which resulted in no significant impact on the overall late summer or autumn flowering response. This has also been reported in previous studies (Burke, 1951, Barbera and Carimi, 1988). The high rate of floral abscission may have been as a result of possible low tree carbohydrate status due to the prolonged period of WD stress (Goldschmidt, 1999), floral damage by insects (Childers, 1992), or floral damage by wind (Davies and Albrigo, 1994).

Expression of *FT*, *AP1* and *AG* was not affected by regulated WD stress during summer, although a non-significant (P = 0.0861, 95% confidence level) increase in *FT* expression was observed in extensively stressed trees compared to untreated control trees, which is in contrast to results from studies by Chica and Albrigo (2013) using 'Washington Navel' sweet orange. Overall, regulated WD stress treatments reduced vegetative vigour during summer compared to untreated control trees, which could be of commercial importance to producers who wish to control tree vigour. Additionally, regulated WD stress had no impact on the quality of the winter crop at time of commercial harvest, which is a result similar to that of Barbera and Carimi (1988) on 'Femminello comune' lemon. We conclude that the *forzatura* technique is locally reproducible but its effects on flowering are inconsistent due to varying climatic conditions and a high rate of floral abscission. Additional studies should be conducted over a longer period and should include evaluation of the quality of the summer, *Verdelli* crop. Different cultivars, including those traditionally selected for use in the *forzatura* technique in Sicily should be included in such studies.

Conclusion

No clear conclusion could be made on the practicality of shifting the current winter lemon supply peak to a summer harvest window. However, the use of GA_3 during winter was highly effective in inhibition of spring flowering, and could successfully reduce the winter crop while simultaneously increasing spring vegetative shoot growth and subsequent potential floral bearing positions of a summer crop. This could be of great benefit to lemon producers with the aim to rapidly increase the rate of tree volume development i.e. filling the space in the row, by favouring vegetative instead of reproductive development. Varying climatic conditions impacted the success of this study in stimulating a considerable late summer or autumn flowering reaction. Therefore, the efficacy of the *forzatura* technique seems to be highly dependent on the specific microclimate. Nonetheless, we observed decreased vigour in lemon trees upon WD stress treatment with no effect on the internal quality of winter fruit, which may have commercial benefit for producers saving on summer pruning costs, as well as decreasing water usage.

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